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PREFACE

This number contains the papers read at a Symposium of the Society for Experimental Biology, which was held at Cambridge in July, 1946. It is the first number of an annual series of symposium reports. Explanations of Plates are given at the end of each paper. Dr R. J. C. Harris has assisted us in the proof reading.

The Symposium for 1947 will be on 'Growth in Relation to Differentiation and Morphogenesis'.

The Society is deeply indebted to Imperial Chemical Industries, Ltd., the Rockefeller Foundation and the British Council for financial aid which made the 1946 Symposium possible. We are also most appreciative of the facilities and help given to us by the British Council and by Cambridge University.

The editors wish further to thank the Cambridge University Press for the kindness and courtesy with which they have assisted us in producing this report.

J. F. Danielli
R. Brown

Honorary Secretaries
Society for Experimental Biology

THE STRUCTURES OF NUCLEIC ACIDS

By J. MASSON GULLAND
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I. INTRODUCTION

The early investigators at the end of last century and beginning of this undoubtedly pictured the 'nucleins' of those days as multi-basic phosphorus-containing acids of molecular weights so large as to prevent dialysis, but with a few notable exceptions (e.g. Hammarsten, 1924; Feulgen, 1935, 1936) developments tended increasingly to follow problems of detailed structure of the nucleotides, or their derivatives, which were isolated from the fission products of nucleic acids.

Two types of nucleic acid only were, and indeed are still, recognized, that in which the carbohydrate is pentose and that in which it is desoxypentose; the existence of hybrid nucleic acids containing both types of sugar has been postulated (Donovan & Woodhouse, 1943), but in the absence of experimental evidence this suggestion is purely speculative (Gulland, Barker & Jordan, 1944). The acid prepared from yeast was regarded as characteristic of the first type, and it was well recognized that the nucleotides which can be isolated from its fission products are guanylic, adenylic, cytidylic and uridylic acids. Typical of the latter type was the acid obtained from thymus gland, the nucleotides being those derived from guanine, adenine, cytosine and thymine (5-methyl uracil). There were many indications of a general assumption that these were the only nucleic acids, common throughout Nature. In order to avoid undue length, the more detailed part of this communication is restricted to discussions of the structure of these two nucleic acids, which have in fact been examined in much greater detail than most other nucleic acids.

Several major factors, outlined below, led to a fairly wide assumption that the molecules of yeast ribonucleic acid and thymus desoxypentose nucleic acid consisted of simple tetranucleotides, structures in which the four appropriate nucleotides were joined by ester linkages between their phosphoryl groups and a sugar hydroxyl of their neighbours:

(i) The close similarity between the breakdown products of the two nucleic acids.

(ii) Demonstration that yeast ribonucleic acid contained equimolecular proportions of purine and pyrimidine nucleotides.

(iii) A frequent assumption that, as the major features of the structures of both acids appeared to be the same, conclusions reached from experiments on one acid could be applied directly to considerations of the other.

(iv) The recording of a molecular weight for yeast ribonucleic acid corresponding roughly to that required for a tetranucleotide.

(v) Data recorded by various investigators of the titration of both acids, either electrometrically or to the phenolphthalein end-point (thus supposedly titrating primary and secondary phosphoryl groups), showed that the acids exhibited approximately either four or five acidic dissociations for four atoms of phosphorus. The presence of five such groups, believed to be four primary and one secondary, could only be explained on the basis of a tetranucleotide of open-chain structure, which could supposedly have arisen by hydrolysis of a cyclic tetranucleotide with four primary phosphoryl groups.

(vi) Preparations of specific enzymes, phosphomonoesterase and phosphodiesterase, failed to cause dephosphorylation of yeast ribonucleic acid when acting separately, whereas a mixture did so; these results favoured the theory of a cyclic tetranucleotide.

With the advent in 1938 of more extensive knowledge of molecular sizes, ideas as to nucleic acid structures reverted sharply to the early conception of large molecules; it became obvious that all nucleic acids are vastly larger than tetranucleotides, and the term polynucleotide came into vogue.

Examination of the literature (Schmidt, Pickels & Levene, 1939; Fischer, Lehmann-Echternacht & Böttger, 1941; Bredereck & Jochmann, 1942; Bredereck & Hoepfner, 1942; Fischer & Lehmann-Echternacht, 1943; Tennent & Vilbrandt, 1943; Fletcher, Gulland & Jordan, 1944; Fletcher, Gulland, Jordan & Dibben, 1944; Ahlström, Euler, Fischer, Hahn & Högberg, 1945) shows clearly that the molecular sizes vary considerably with the type and sources of the nucleic acids and also with their methods of isolation and purification; this point is treated in more detail by Gulland & Jordan (1946), and it is sufficient to emphasize here that all nucleic acids are susceptible to alkaline conditions whether mild or drastic, and that the latter undoubtedly causes degradation through hydrolysis. As regards the desoxypentose nucleic acid of thymus, Signer, Caspersson & Hammarsten (1938) recorded a molecular weight of between 500,000 and 1 million, that is, between about 1500–3000 nucleotides; Astbury & Bell (1938) confirmed this size as being about 2000 nucleotides; and Tennent & Vilbrandt (1943), and Schmidt *et al.* (1939) quoted molecular weights of 500,000 and at least 1–2 millions respectively. Turning to the pentose nucleic acids, Loring (1939*a*) found molecular sizes of 116 nucleotides for the acid of tobacco mosaic virus and 56–88 nucleotides for the acid of yeast. Cohen & Stanley (1942) found an approximate particle weight of 300,000 for the freshly isolated nucleic acid of tobacco mosaic virus. Fischer, Böttger & Lehmann-Echternacht (1941) recorded a weight of 10,350 for the acid of yeast, whereas Fletcher, Gulland, Jordan & Dibben (1944) found a range between 10,280 and 23,250, depending on the commercial source of the samples and hence possibly on their treatment during extraction.

This recognition of the complex character of nucleic acids undoubtedly opened afresh the whole question of their structures (Gulland, 1944), but it is surprising to observe how readily there has occurred a mental superposition of the newer knowledge of molecular sizes on to the older ideas of the simple tetranucleotides. Although the term tetranucleotide originally expressed the apparent occurrence in approximately equimolecular proportions of the four nucleotides, or frequently of the corresponding nitrogenous derivatives after fission, it came later to be used by some workers to denote a unit which by uniform repetition (polymerization or polycondensation) builds up the polynucleotide molecule as a polytetranucleotide. Further, as an extension of this conception have come postulations that in each of these units the four nucleotides are invariably combined in a fixed manner in an unchangeable sequence. The term tetranucleotide has thus in different minds a graded series of implications, ranging from a statistical expression of analytical results to the definition of an exact, uniform chemical entity; in order to avoid confusion in discussion the terms statistical and structural tetranucleotide have been suggested (Gulland, Barker & Jordan, 1945).

These questions are touched on more fully below, but it is opportune to state here that in our opinion there is at present no indisputable evidence that any polynucleotide is composed largely, if at all, of uniform, structural tetranucleotides; this is not to aver that such may not be discovered in the future, but rather to emphasize the present need for maintaining an open mind.

It will be shown later that polynucleotides of medium to high molecular weights consist of nucleotides united substantially or entirely through phosphoester linkages; in other words, the linkages between structural tetranucleotides, if such exist, can only be of the same type as those uniting the component nucleotides. Moreover, all determinations of tetranucleotide sizes, properties and compositions, all estimates of purine-pyrimidine ratios, are based on experimental procedures which yield average values only. It follows, therefore, that there is no chemical reason why individual nucleotides should necessarily occupy positions in a regular sequence in the polynucleotide chain; there is, to choose perhaps an extreme case as illustration, no reason why four molecules of a given nucleotide should not be adjacent and be succeeded in the chain by, let us say, a group of molecules of another nucleotide. These possibilities weaken considerably the concept of the structural polytetranucleotide, which is further undermined by demonstrations that although some acids do, others do not, conform to the statistical requirements of a tetranucleotide. Thus, although yeast ribonucleic acid is approximately a statistical tetranucleotide (see below) and the pentose nucleic acid of sheep liver (Davidson & Waymouth, 1944*a*) contains purine and easily hydrolysable phosphorus in amounts consistent with the tetranucleotide ratio, the pentose nucleic acid of tobacco mosaic virus has a purine content 20% lower than that required (Loring, 1939*b*), the pentose polynucleotide of pancreas is recorded as containing a considerable, if variable, excess of guanylic acid (Jorpes, 1928, 1934; Levene & Jorpes, 1930; Steudel, 1936), the pentose nucleic acid of rat liver has a nitrogen-phosphorus ratio which exceeds that required (Brues, Tracey & Cohn, 1944), and the desoxypentose nucleic acid of calf thymus (see below) is slightly deficient in purines, which are replaced by pyrimidines.

It is evident that on the basis of the views outlined above, and unless and until these are disproved beyond doubt, claims that two nucleic acids are identical cannot be entertained unless these possibilities of isomerism are taken into account; they may exhibit the same physical properties and have the same analytical composition, but, nevertheless, be chemically and biologically distinct. The possibilities of biological specificity of nucleic acids are clearly very extensive; it may therefore eventuate that if nucleoproteins are found to be specifically characteristic, whether of large biological groups, individuals or even particular organs, or of healthy and diseased tissues, etc., both nucleic acid and protein may contribute to the differentiation.

II. YEAST RIBONUCLEIC ACID

This acid has been examined more thoroughly than any other pentose nucleic acid. It should be realized, however, that so far as is known all samples which have been studied in any detail have been isolated by processes involving treatment with alkali, a reagent to which the acid is remarkably susceptible. It is therefore questionable as to how far these samples are representative of the acid in its native state. At best it seems likely that the acid may have undergone hydrolysis, the extent of which probably depends on the severity of the extraction treatment; as mentioned above, preparations from different sources certainly have different molecular sizes. There are, however, further complications, since hints are not lacking that samples of the acid may not always have identical structures; lability to alkaline hydrolysis is a variable property (Steudel, 1930; Makino, 1935*a*; Zittle, 1946*b*), ribonuclease activity alters considerably with the sample of nucleic acid (Kunitz, 1940; Zittle, 1946*b*) and is also sensitive to the age of the substrate solution, and only certain preparations of the acid yield guanine-uridylic acid on hydrolysis (Falconer, Gulland, Hobday & Jackson, 1939; Gulland, 1940; Bredereck, Berger & Richter, 1941). Until satisfactorily explained (see, for example, Zittle, 1945, 1946*a*), such questions must tint all reflexions on the structure of yeast ribonucleic acid.

Cold alkaline hydrolysis of the acid yields a solution from which can be isolated, although only in far from quantitative yields, the four nucleotides guanylic, adenylic, cytidylic and uridylic acids. Every detail but one of their constitutions is now settled beyond doubt; the earlier work, due so much to Levene and his collaborators, was reviewed by Gulland (1938), and the position of the phosphoryl groups in the pyrimidine nucleotides, then left undetermined, has now been established at C_3 of the sugar by Gulland & Smith (1946). A decision on the remaining question, the configuration of the glycosidic linkages, is predicted by Lythgoe & Todd (1944). Doubts which surrounded Levene's identification of the carbohydrate groups as *d*(–)-ribose have now been resolved (Gulland & Barker, 1943; Barker, Farrar & Gulland, 1946) and the original conclusion confirmed.

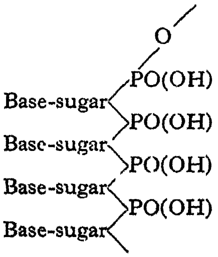
The acid contains purine and pyrimidine nucleotides approximately in the tetranucleotide ratio (Levene, 1909; Jones, 1920; Hoffman, 1927; Ellinghaus, 1927; Kobayashi, 1932; Gulland, Jordan & Threlfall, 1946), but the exact proportions of each nucleotide are unknown.

Yeast ribopolynucleotide is the only pentose nucleic acid which has as yet been considered seriously from the standpoint of a structural tetranucleotide. Milder conditions of hydrolysis than those which effect complete fission into nucleotides yielded a product considered by Bredereck & Hoepfner (1942) to be a structural tetranucleotide. This and the corresponding deaminated material (NH_2 replaced by OH) were approximately penta-basic acids of the correct analytical compositions and having molecular weights of 1177 and 1121 (calculated 1304 and 1307). It has been pointed out that these materials cannot have been tetranucleotides (Fletcher, Gulland & Jordan, 1944); the deaminated acid should have been hexa-basic, since the hydroxyl dissociation of xanthine titrates over the range *p*H 6–8 and its presence in deaminated yeast ribonucleic acid was demonstrated by electrometric titration. Moreover, the low molecular weights claimed for these materials could not be confirmed (Fletcher, Gulland, Jordan & Dibben, 1944), and it was concluded that there is no evidence on these grounds for the existence of a structural tetranucleotide. Exact relative positions of nucleotides were defined by detection of individual nucleotides in the early stages of enzymic fission (Bolomey & Allen, 1942) or aqueous pyridine hydrolysis (Bredereck *et al.* 1941) of the acid, but in our opinion such results indicate merely that some linkages in the polynucleotide are more labile than others and hence permit more rapid liberation of some nucleotides.

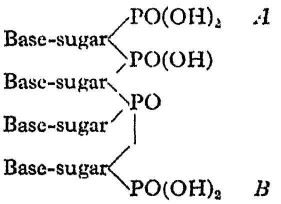
A study of ribonuclease (ribonucleinase), the heat-stable pancreatic enzyme believed to be specific for ribopolynucleotides and hence finding employment in their histological demonstration (Brachet, 1940*a*, *b*; Deselin, 1940; Painter & Taylor, 1942; Gersh & Bodian, 1943; Davidson & Waymouth, 1944*b*), seemed at one time to offer prospects of its use in the preparation of a structural tetranucleotide in view of its liberation of one secondary phosphoryl dissociation for every four atoms of phosphorus. Further investigation has shown, however, that its action is more complex; the average size of the dialysable products from the action of preparations of heat-stable pancreatic enzymes in the nucleic acid corresponded to that of a dinucleotide (Fischer, Böttger & Lehmann-Echternacht, 1941), but according to Loring & Carpenter (1943) all four nucleotides are isolated from the products of digestion with crystalline ribonuclease. Nevertheless, the exact effect of the enzyme is still not clear (Zittle, 1946*b*). It is generally agreed that its action renders about half of the phosphorus of the acid non-precipitable by the uranyl-trichloracetic acid reagent (MacFadyen, 1934) although still organically combined; the remainder of the molecule resembles the original nucleic acid in being precipitated by this reagent and also by hydrochloric acid (Schmidt & Levene, 1938). It should not be inferred that this precipitable material is necessarily unchanged nucleic acid. Loring (1944) has pointed out that yeast ribonucleic acid must contain at least two different types of linkage, one labile, the other resistant, to the action of ribonuclease, and it is not clear whether the liberated nucleotides come equally from every nucleic acid molecule and whether they are removed by transverse fission of the polynucleotide chain or are lopped from a main polynucleotide trunk bearing nucleotides, or nucleotide chains, as branches (see below). The nature of ribonuclease action is rendered even more obscure by the demonstration (Cohen, 1945) that even the crystalline enzyme exerts proteolytic action.

The question of internucleotide linkages must now be considered. There is not the slightest doubt that the phosphoryl groups are involved, although not as pyrophosphate linkages, and since it has been shown (Fletcher, Gulland, Jordan & Dibben, 1944) that the action of nitrous acid on the nucleic acid is to yield a polynucleotide of undiminished molecular weight, the amino groups cannot take part in these linkages to any substantial extent. This conclusion was confirmed by the results of electrometric titration (Fletcher, Gulland & Jordan, 1944), which also revealed the unsubstituted state of two purine-pyrimidine hydroxyl groups for each four atoms of phosphorus, that is, those of guanine and uracil. It follows that in the main the internucleotide linkages must be phospho-ester groups, and the hydroxyls at C_2 and C_5 of the carbohydrates are alone available for these, since that at C_3 is esterified by phosphoryl in the nucleotides and that at C_4 is engaged in the furanose ring. This conclusion introduces the problem that yeast ribonucleic acid is readily hydrolysed by cold 1% sodium hydroxide, whereas the phosphoryl groups of nucleotides and of related 5-phospho-esters (e.g. muscle adenylic acid) are stable in these circumstances. Levene & Tipson (1935*a*) suggested that the alkali-sensitive groups are situated at C_2, drawing support from a supposed lability of substituents at C_2 of carbohydrates in general, and their proposal seems to have been generally accepted (see, for example, Makino, 1935*a*; Bredereck, 1938). Nevertheless, the literature does not record for substituents in the hydroxyls of sugars differences in stability so marked in degree as to explain the complete fission of an ester at C_2 whilst a similar linkage at C_3 remains unattacked (Gulland & Walsh, 1945), and uridine-2-phosphate, synthesized by procedures which leave no doubt as to its constitution (Gulland & Smith, 1946), is not dephosphorylated by cold sodium hydroxide and has a stability towards hot alkali of the same order as that of uridylic acid. It seems, therefore, that the ready fission of the nucleic acid by alkali is a property of the polynucleotide rather than of ester groups at particular positions, and support for this view can be found in the facile alkaline hydrolysis of di- and tri-esters of phosphoric acid as compared with mono-esters (e.g. Bailly & Gaumé, 1926; Plimmer & Burch, 1929); nevertheless, other factors must also operate, since desoxypentose nucleic acids, which also contain phospho-diester linkages, are far more stable towards alkali. This conclusion opens the possibility that, in addition to that at C_3, hydroxyl groups at C_2 or C_5, or at both, may be involved in internucleotide linkages and raises the question whether nucleotides other than those already recorded occur among the fission products of the polynucleotide. These are matters for investigation.

In view of the complete agreement that yeast ribonucleic acid exhibits four phosphoryl dissociations for each four atoms of phosphorus, and since hydrolysis results in the appearance of secondary phosphoryl groups, it might be assumed that the polynucleotide is an unbranched chain composed of a succession of tetranucleotides of the structure (I) proposed by Levene & Simms (1926), and that in consequence each of its phosphoryl groups should exhibit one primary dissociation, except the terminal phosphoryl with one primary and one secondary. This view is untenable, as was shown by electrometric titration between *p*H 2 and 12 (Fletcher, Gulland & Jordan, 1944) of the nucleic acid and its deaminated derivative, prepared by a method which avoided any fall in molecular weight. Comparison of the experimental curves with those constructed theoretically showed that for each four atoms of phosphorus there are three primary and one secondary phosphoryl dissociations.* If these observations are considered on the basis of a polytetranucleotide, merely as a convenient standard for assessment, each individual unit of the polymer would contain one triply esterified, and therefore neutral, phosphoryl group, two phosphoryls each exhibiting one primary dissociation, and one phosphoryl singly linked to a carbohydrate radical and having both a primary and a secondary dissociation (II).

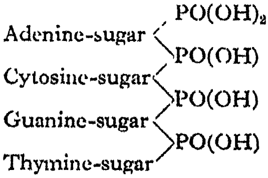


I



II

Polynucleotide condensation either through *A* or *B*, the other phosphoryl remaining singly linked



III

Without necessarily assuming a uniform structural tetranucleotide, it is possible that constitutional relationships of the type indicated may occur in the polynucleotide, and two points are noteworthy. First, the literature frequently reveals low figures in phosphorus analyses of yeast ribonucleic acid. In an unbranched polynucleotide a deficiency of phosphorus could only occur through absence of the singly linked terminal phosphoryl, but the extent of the deficiency is often greater than can be accounted for in that way. In a polynucleotide comprised of structures such as those just outlined, up to 25% of the total phosphorus could be absent without upsetting the main polynucleotide. Secondly, a molecule can be visualized in which nucleotide side-chains could be removed by the action of ribonuclease, leaving a molecule sufficiently large to resemble the original nucleic acid in its failure to dialyse and in its precipitation reactions.

III. THYMUS DESOXYPENTOSE NUCLEIC ACID

Chemical hydrolysis failed to dismember this acid satisfactorily, and Klein & Thannhauser (1933, 1934, 1935; see also Brady, 1941) attacked it with enzymes of intestinal mucosa, and isolated the desoxypentose nucleotides

* After this communication had been printed, I received copies of papers by Khouvine and Grégoire (*Bull. Soc. chim. biol.*, 1944, **26**, 421, 424) on the pentose nucleic acid of the larvae of *Calliphora erythrocephala*; this acid exhibits four phosphoryl dissociations, of which one is secondary, for each four atoms of phosphorus (corrected for the phosphorus analysis), and thus resembles yeast ribonucleic acid in this respect. J. M. G.

related to guanine, adenine, cytosine and thymine, which they dephosphorylated to the corresponding nucleosides. Up to the present other products of this enzymic fission have not been recorded, but little search seems to have been made.

Knowledge of the structures of these breakdown products is as yet rather incomplete. The positions of the phosphoryl groups are unknown; it is practically certain that they are at C_3 or C_5 of the sugar radicals, but it is not known whether the position is the same for all the nucleotides. The positions of the sugars in the purine radicals were shown (Gulland & Story, 1938*a*, *b*) by measurements of ultra-violet absorption spectra, but in the pyrimidine nucleosides the positions are guessed by analogy with the pyrimidine nucleosides of yeast ribonucleic acid. It seems to be widely accepted that the sugar (thyminose) is *d*-2-desoxyribofuranose in each of these nucleosides and in desoxypentose nucleic acids in general. This may indeed be the case, but it should be realized that at present the assumption of furanose structure is based on demonstration of its presence in the thymine nucleoside (Levene & Tipson, 1935*a*, *b*; Makino, 1935*b*), and that the identification of the sugar itself applies only to the guanine nucleoside of the acid from thymus (Levene & London, 1929; Levene & Mori, 1929; Levene, Mikeska & Mori, 1929–30); the sugars of the other nucleosides and of desoxypentose nucleic acids from other sources do not seem to have been prepared, and colour reactions such as those of Feulgen or Dische, although commonly regarded as specific for desoxypentose, only demonstrate in reality the presence of a desoxy-sugar.

Despite the fact that desoxypentose nucleic acids are labile towards alkali, many investigators employ sodium hydroxide in their extraction (Levene, 1921, 1922; Stern & Willheim, 1934; Klein & Beck, 1935; Vowles, 1940; Ahlström *et al.* 1945). Examination of the molecular sizes (Schmidt *et al.* 1939; Cohen, 1942; Tennent & Vilbrandt, 1943) of samples of the thymus polynucleotide prepared by different methods showed that alkaline treatment or the action of heat causes diminution as compared with those of materials obtained by milder procedures in neutral solutions and at low temperatures. It is thus clear that mildness of extraction and purification is a vital factor in all studies of desoxypentose nucleic acids. The conclusion has been drawn (Gulland *et al.* 1945) that, where possible, nucleic acids should be prepared by isolation and subsequent decomposition of the nucleoprotein, which can readily be obtained by the method of Mirsky & Pollister (1942, 1943). Remarkably simple preparations of the desoxypentose nucleic acids of calf thymus and rat liver are those in which the protein fraction is denatured and precipitated by sodium chloride or removed by the technique of Sevag, Lackman & Smolens (1938), and the nucleic acid, apparently little if at all degraded, is then precipitated by alcohol (Bang, 1903; Hammarsten, 1924; Greenstein & Jenrette, 1940; Brues *et al.* 1944; Gulland, Jordan & Threlfall, 1946).

The considerations just outlined clearly affect discussions of the statistical tetranucleotide ratio, and earlier results bearing on this question are probably unreliable. Recently, Mirsky (1943) has stated, on the basis of the Dische diphenylamine colour reaction which can be made specific for the purine, as distinct from the pyrimidine, nucleotides, that all desoxypentose nucleic acids so far examined, but from sources as yet unnamed, agree in having equimolecular proportions of total purine and pyrimidine nucleotides; the method does not distinguish between individual nitrogenous radicals. In the case of the desoxypentose nucleic acid of thymus, prepared in a highly polymerized state by methods in which the reaction was maintained neutral, Gulland, Jordan & Threlfall (1946) find that the purine pyrimidine ratio is low as compared with that of a statistical tetranucleotide, some of the adenine being replaced by cytosine (see below).

The actions of enzymes and of hot alkali on desoxypentose nucleic acids have yielded materials which have been regarded as structural tetranucleotides. Thus pancreatic thymopolynucleotidase liberated one acid equivalent (titrated to *p*H 9) for each four atoms of phosphorus and the products, named oligonucleotides, corresponded to 3·9 nucleotides in size (Fischer, Lehmann-Echternacht & Böttger, 1941; Fischer, Böttger & Lehmann-Echternacht, 1941; Fischer & Lehmann-Echternacht, 1943). Oligonucleotides corresponding to 3·2–4·0 nucleotides in size, together with material of higher molecular weight, were also obtained by degrading the nucleic acid with hot alkali, and drastic alkaline extraction and purification of the desoxypentose nucleic acid of beef spleen yielded material considered to be closely similar to the oligonucleotides, in that it had approximately the correct analytical composition, acidity and molecular weights for molecules composed of four nucleotides (Bredereck & Jochmann, 1942; Bredereck & Hoepfner, 1942). It has been postulated by Fischer and by Bredereck that the oligonucleotides are true structural tetranucleotides of the constitution proposed by Levene & London (1929). Definite positions were assigned (Fischer, 1942) to the nucleotides of adenine and thymine, the relative orientation of the others being undetermined, on the basis of the following experiments (III). A mixture of mononucleotidase and oligonucleotidase (phospho-diesterase) from intestinal mucosa rapidly splits off 25% of the total phosphorus from the oligonucleotides. Further dephosphorylation then occurred, and when this had reached 50% the phosphorus-free fraction contained only one molecular proportion of nucleoside, consisting of a mixture of about equal parts of the nucleosides of adenine and thymine.

Neither guanine nucleoside nor mononucleotides could be isolated from the solution. This evidence cannot be regarded as unequivocal (Gulland *et al.* 1945) and does not justify the recognition of the existence of the structural tetranucleotide, whatever may eventuate in the future.

The value of electrometric titration in the study of the internucleotide linkages of yeast ribonucleic acid suggests the application of the same procedure in the case of thymus desoxypentose nucleic acid. Hitherto, conflicting results have been obtained in investigations of the acid-base properties of this acid (Steudel, 1912; Feulgen, 1919; Levene & Simms, 1925, 1926; Makino, 1935*a*, *b*; Bredereck, Köthnig & Lehmann, 1938; Bredereck & Köthnig, 1939; Ahlström *et al.* 1945), and these may be ascribed to the different degrees of degradation of the samples studied. Perhaps the least degraded specimen of this acid as yet examined from this standpoint was that prepared by the method of Bang (1903) and studied conductimetrically by Hammarsten (1924) and electrometrically by Jorpes (1934). It was, however, very deficient in nitrogen and phosphorus (N, 11·97; P, 7·09 %) as compared with the theoretical requirements of the tetrasodium salt (N, 15·85; P, 9·37 %).

The highly polymerized nucleic acid (actually the tetrasodium salt) prepared in neutral conditions by Gulland, Jordan & Threlfall (1946) was examined electrometrically by Gulland, Jordan & Taylor (1946), and also the thymic acid prepared from it by the action of warm, dilute mineral acid; this causes marked fission of the molecule and removes specifically the whole of the guanine and adenine radicals. The initial titration of the nucleic acid between *p*H 2·5 and 12 is abnormal, and is discussed by Gulland & Jordan (1946). On back-titration either with acid from *p*H 12 or with alkali from *p*H 2·5, a normal titration curve is obtained, and analysis of this on the basis of a tetranucleotide, for convenience of presentation, coupled with the evidence deduced from the titration curve of thymic acid, led to the following conclusions:

	Primary phosphoryl	Secondary phosphoryl	Amino	Purine-pyrimidine hydroxyl	Sugar hydroxyl
Desoxypentose nucleic acid	$\nless$ 3·9	$\ngtr$ 0·1	3*	2†	0
Thymic acid	4·0	0·8	1·1–1·2‡	1§	+

* 1 guanine, 2 made up of adenine and cytosine.
† 1 guanine, 1 thymine.
‡ Cytosine.
§ Thymine.

These data are consistent with the view that thymus desoxypentose nucleic acid has a long-chain structure (Signer *et al.* 1938; Astbury & Bell, 1938), and that branching of the chain, as observed in the case of yeast ribonucleic acid, does not occur to any appreciable extent in view of the almost entire lack of secondary phosphoryl dissociation. The absence of sugar hydroxyl dissociation, the presence of one primary phosphoryl dissociation for each atom of phosphorus, and the titratability of the amino and hydroxyl groups of purines and pyrimidines, supports the view that the internucleotide bond is an ester linkage. The titration curves give additional information concerning the proportions of the four nitrogenous radicals and confirm the observation (above) that although guanine and thymine are present in molecular ratio, adenine is deficient and is replaced by cytosine. The presence of 0·8 equivalent of secondary phosphoryl dissociation for every four atoms of phosphorus of thymic acid, or one equivalent to every five atoms of phosphorus, suggests, on the basis of a straight-chain formula, that the average number of nucleotides in thymic acid is five, the average molecular weight being about 1200.

IV. CONCLUSION

There is at present no indisputable chemical evidence that the nucleotides are arranged in other than a random manner in the polynucleotides. It must be realized that the existence of tetranucleotide units, repeated throughout the molecule, would limit the potential number of isomers and hence diminish the possibilities of biological specificity. Thus, the immense number of variations presented by a polynucleotide in which the nucleotides occur in random sequence is reduced, in the case of an unbranched polynucleotide, to a single possible structure, varying only in its length, if the same, uniform tetranucleotide unit occurs throughout.

It is a pleasure to record my gratitude to my colleague, Dr D. O. Jordan, with whom I have discussed many points in this communication.

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STRUCTURE AND SYNTHESIS OF NUCLEOTIDES

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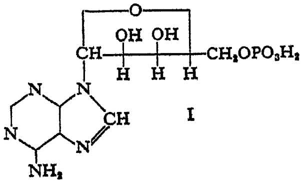
University Chemical Laboratory, Cambridge

Degradation and synthesis are complementary methods for the elucidation of molecular structure, and in many groups of naturally occurring substances the use of synthetic methods has led to a knowledge of constitutional features much more rapidly and completely than would otherwise have been possible. The nucleic acids are exceedingly complex structures, so that total synthesis by chemical methods may well seem at present beyond the bounds of possibility; nevertheless, we believe that synthetic studies in this field may ultimately prove of great value in furthering an understanding of the structures and mode of biological action of these substances, and of the smaller but closely related molecules whose importance has become clear during the last decade.

Among the latter are the coenzymes of phosphate transfer derived from muscle adenylic acid (I) such as adenosine triphosphate, which are mononucleotides and belong to the same class as the simple nucleotides derived from hydrolytic breakdown of the macromolecular nucleic acids; that is, they are phosphate esters of a sugar in glycosidic combination with a base molecule derived from pyrimidine or purine. Rather more complex substances of similar structure are the coenzymes of hydrogen transfer such as cozymase (II) and triphosphopyridine nucleotide, in which two such nucleotide units are united by a polyphosphate residue; flavine-adenine dinucleotide, although not strictly speaking a dinucleotide, may be considered as belonging to this class. The nucleic acids themselves have been shown by recent work to contain a very large number of nucleotide units, justifying the name of polynucleotides.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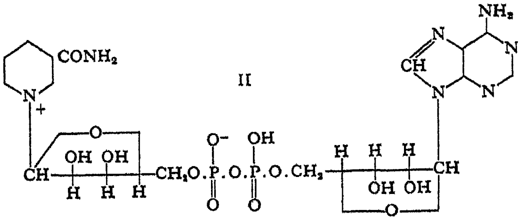

II

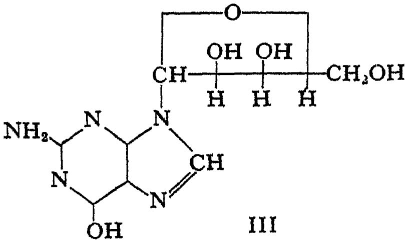
No justification is needed for structural investigation of this large and biologically important class of compounds. The mononucleotides, of which four, each containing *d*-ribose as sugar component, are obtained from ribonucleic acids, and four different units, each containing *d*-2-deoxyribose, are derived from deoxyribonucleic acids, have been studied by degradative methods for the past 30–40 years. Although the main outlines of their structures are now clear, certain important features still remain to be definitely established. Exact knowledge in this part of the field seems all the more desirable, since it must form the basis for all suggestions regarding the structures of the polynucleotides. The structures accepted as representing the coenzymes have also been deduced from degradative investigations. Relatively little is known of the chemistry of these compounds, for the pure substances are difficult to obtain in quantity, and no structural analogues are accessible, so that the biological specificities of the compounds and the nature of their linkages with different protein apoenzymes are not understood. Since the same problems arise in the case of the plant viruses and similar nucleoproteins endowed with autosynthetic activity, information on these points for the simpler systems would be of great value.

In order to throw light on these problems, we and our collaborators determined to investigate the possibilities of total synthesis in this field. Such an approach would require the development of general and flexible synthetic methods, of a type which would preferably demonstrate the constitution of the products so obtained, and would start with the smallest units (nucleosides) and build up to the larger and more complex structures. The problems requiring consideration in a synthetic approach on the widest possible basis are seen to fall into three groups: (*a*) synthesis of nucleosides, (*b*) phosphorylation of sensitive molecules (nucleotide synthesis), (*c*) linkage of molecules through phosphate and polyphosphate residues (polynucleotide synthesis). In our investigations, an account of which we shall give in the present contribution, most attention has naturally been devoted to the first two problems up to the present, but some indications will be given in turn of the progress made in each of the three fields. Some results which might follow from successful solutions to these problems may first be considered.

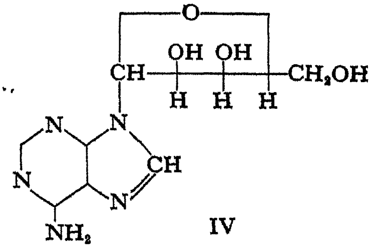
So far as concerns the mononucleotides one might reasonably expect in this way to obtain complete clarification of all outstanding structural issues, and valuable insight into the chemical properties of the compounds as a class. This might be of use in throwing light on the nature of the internucleotidic linkage in nucleic acids; for example, the behaviour of the various monophospho- and diphospho-nucleosides towards hydrolytic agents should reveal why only 3′-phosphonucleosides are obtained from hydrolysis of polynucleotides. Amongst the coenzymes, the possession of adequate synthetic methods would allow the production of variants of the natural molecules for use in biological studies, where they might act by a competitive mechanism. In the polynucleotide field, synthetic methods would be of most value when coupled with degradative methods of a more specific nature than those which up till now have been the main means for their investigation. Ideally, one would wish to isolate from breakdown of a polynucleotide fragments which while small enough to permit isolation and examination by available techniques are yet large enough to retain and reveal some of the structural features of the parent molecule. In this respect the work of Bredereck on the breakdown of ribonucleic acids (Bredereck, Berger & Richter, 1941; Bredereck & Hoepfner, 1942), and of deoxyribonucleic acids (Bredereck & Jochmann, 1942) in which the isolation of homogeneous oligonucleotides containing three or four units was claimed, may, although it has not itself been confirmed (Fletcher, Gulland & Jordan, 1944), indicate the direction in which progress may be expected in the future. At present, stepwise degradation by chemical methods is difficult because of the similarity of the various linkages involved, but this should be surmountable when pure enzyme preparations become available. Both chemical and enzymatic approaches would be greatly furthered if it were possible to study the behaviour of oligonucleotides of known structure. The value of such methods has already been proved in the related field of protein chemistry, both as a means of facilitating isolation and identification of breakdown fragments, and also for purposes of enzyme specificity studies.

I. NUCLEOSIDES

Structure. Before describing the synthetic studies it will be desirable to review briefly the status of structural knowledge of these compounds at the outset of our investigations (Bredereck, 1938; Gulland, 1938, 1944; Lythgoe, 1944; Tipson, 1945). The most fully investigated members are those obtained by hydrolytic dephosphorylation of yeast nucleic acid, viz. guanosine (III), adenosine (IV), cytidine (V) and uridine (VI). Early investigations, largely due to Levene and his collaborators (see Levene & Bass, 1931), had shown that these were ring-N-glycosides; the structures of the aglycones and the identity of the sugar component with *d*-ribose (Gulland & Barker, 1943; Barker, Cooke & Gulland, 1944) had been established. The latter is present in the furanose condition, the most convincing proofs resting on application of the classical procedures of methylation and hydrolysis (Levene & Tipson, 1932, 1933). The pyrimidine nucleosides are N_3-glycosides since uridine can be converted into an N-methyluridine which gives on hydrolysis N_1-methyluracil (Levene & Tipson, 1934*a*). No chemical method was available for determining the location of the ribofuranose residue in adenosine and guanosine, but the N_9 position has been generally preferred to the N_7 position on the basis of spectroscopic evidence brought forward by Gulland and his associates (Gulland, Holiday & Macrae (1934); Gulland & Holiday (1936); Gulland & Story (1938*b*)). Direct verification of this point appeared desirable. One further feature awaited clarification, namely, the configuration (α, β nature) of the glycosidic linkage in the nucleosides. This feature must be of considerable importance in the chemistry of nucleotides, e.g. in determining molecular packing in the polynucleotides and the attachment of coenzymes and polynucleotides to their respective protein partners, but no evidence relating to the problem appears so far to have been presented.



III



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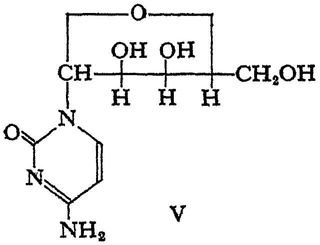

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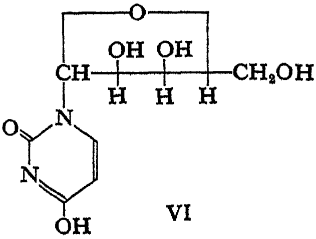
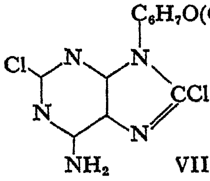


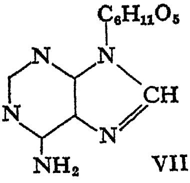
VI

The four nucleosides obtained by breakdown of deoxyribonucleic acids are usually formulated as the 9-*d*-2′-deoxyribofuranosides of guanine and adenine and the 3-*d*-2′-deoxyribofuranosides of cytidine and thymine (5-methyluracil). It must suffice here to indicate in what respects these formulations still await rigid verification. The identity of the deoxypentose component in the pyrimidine members is based on analogy with the purine members. Spectroscopic evidence exists indicating that the purine members are N_9-glycosides (Gulland & Story, 1938*a*, *b*), but the allocation of the sugar to N_3 in the pyrimidine members is justified only by analogy with the corresponding ribonucleosides (Bredereck, Müller & Berger, 1940; Lythgoe, 1944, p. 204). Similar arguments make likely the furanose nature of the sugar component in the deoxyribosides; the experimental evidence so far obtained on this point is not conclusive (Lythgoe, 1944, p. 205). As in the ribonucleosides the stereochemistry at the glycosidic centres is unknown.

Synthesis. On account of its participation in coenzyme molecules, adenosine is probably the most important nucleoside, and it was therefore to the synthesis of this that our studies were in the first place directed. Other workers have in the past devoted some attention to this problem, and in fact one method which appears to be feasible for such a synthesis has been found. Fischer & Helferich (1914) showed that acetohalogeno sugars react with suitable purine derivatives, the products obtained by removal of acetyl groups being true N-glycosides. A wide variety of theophylline-7-glycosides (for evidence on the N_7 location of the sugar residue see Gulland, Holiday & Macrae, 1934) has been synthesized in this way, and they have proved useful as models for investigating the behaviour of purine glycosides. By using as starting materials 2:8-dichloroadenine and acetobromoglucose, a tetra-acetylglucoside (VII) is obtained from which by deacetylation and reductive dehalogenation an adenine glucoside (VIII) is formed. From spectroscopic evidence this appears to be a 9-glycoside (Gulland & Story, 1938*a*; Gulland, 1938). Completion of a synthesis of adenosine by this route would demand as an intermediate an acetohalogenoribofuranose, and no compounds of this type have so far been described in the literature. Moreover, this method of synthesis leaves in doubt the position occupied by the sugar residue in the products so obtained, and it seems to offer only limited scope for the preparation of glycosides bearing substituents in the purine nucleus. For our purpose therefore this method would be of little value.



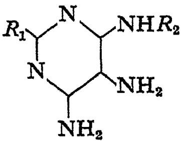
VII



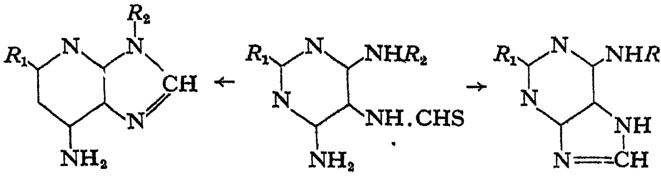
VIII

A survey of the methods which can be used for synthesis of purine derivatives shows that the method used by Traube (1904) is the most versatile, and we decided to study the possibility of modifying this method to allow its application to 9-glycosidopurines. The essential intermediate in such a synthesis would be the 5:6-diamino-4-glycosidaminopyrimidine derivative (IX; $R_2 = C_5H_9O_4$ or $C_6H_{11}O_5$). In Traube's synthesis an analogous sugar-free triaminopyrimidine (IX; $R_2 = H$) is heated with formic acid to give the corresponding 5-formamido compound which at higher temperatures loses water and is cyclized to the purine (X; R_2 = H). Clearly such a method is too drastic for our purpose, where all risk of hydrolysis of the sensitive glycosidic linkage must be avoided, so our first task was to develop some milder method of cyclization. This was discovered as a result of experiments in which sugar-free pyrimidine derivatives were used as model substances (Baddiley, Lythgoe, McNeill & Todd, 1943). It was found that 4-amino-5-thioformamidopyrimidines lost hydrogen sulphide when heated in anhydrous pyridine, with cyclization to the purine; for example, 4:6-diamino-5-thioformamidopyrimidine (XI; $R_1 = R_2$ = H), readily prepared by the action of aqueous sodium dithioformate on (IX; $R_1 = R_2$ = H), gave adenine (X; $R_1 = R_2$ = H) in excellent yield (Baddiley, Lythgoe & Todd, 1943*a*).

To prepare a 9-substituted adenine derivative by this method an intermediate (XI) is required, and where *R* is any atom or group other than hydrogen, ring closure of this may theoretically occur in two different ways,



IX

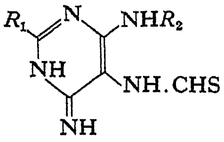


X XI XII

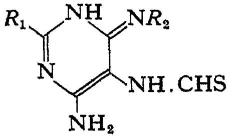
only one of which leads to the desired product (X). A desire for guidance as to which course the reaction would be likely to follow when a glycosidaminopyrimidine derivative (XI; $R_2 = C_5H_9O_4$ or $C_6H_{11}O_5$) was used led us to undertake model experiments on derivatives of type (XI), where R_2 is an alkyl residue. The alternative products in this case are a 9-alkyladenine derivative (X; R_2 = Alk.) and a 6-alkylaminopurine derivative (XII; R_2 = Alk.). The factors determining this and many other aspects of the behaviour of purine and pyrimidine derivatives are the possibilities of tautomerization within the nucleus. From general experience of these compounds it was thought likely that a compound (XI; R_2 = Alk.) would tend to behave in the iminodihydropyrimidine form (XI*a*) rather than as the alternative alkyliminodihydro form (XI*b*). In this event cyclization would involve the alkylamino group rather than the amino group, and formation of (X; *R* = Alk.) should occur preferentially. The validity of this reasoning was borne out experimentally. It was found, for example, that (XI; R_1 = MeS, R_2 = Me) gave rise on cyclization to (X; R_1 = MeS, R_2 = Me) exclusively and in good yield (Baddiley, Lythgoe, McNeill & Todd, 1943).

Since it was anticipated that a glycosidamino group at position 4 in the pyrimidine nucleus would behave in this respect similarly to an alkylamino group, this method seemed of promise for the synthesis of a 9-glycosido-adenine derivative.

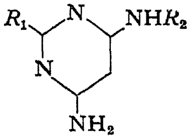
Our attention was next directed towards methods for the preparation of the 5:6-diamino-4-glycosidaminopyrimidine derivative (IX; $R_2 = C_5H_9O_4$ or $C_6H_{11}O_5$) required for such a synthesis. An examination of the possible alternative routes made it clear that the best hopes of success would be offered by initial preparation of a 6-amino-4-glycosidaminopyrimidine derivative (XIII; $R_2 = C_5H_9O_4$ or $C_6H_{11}O_5$), followed by introduction of an amino group into position 5 of the nucleus. Some investigation was found necessary before success in the preparation of glycosides of type (XIII; $R_2 = C_5H_9O_4$ or $C_6H_{11}O_5$) was achieved. Preparation of such N-glycosides by the usual methods (interaction of sugar or acetohalogeno-sugar with the base) is impeded by the fact that an amino group at positions 4 or 6 in the pyrimidine nucleus shows greatly reduced amino behaviour, since tautomerization causes, for example, (XIV) to react mainly in the iminodihydropyrimidine form (XIV*a*). It seemed possible to overcome this difficulty by employing for glycosidization a 4:6-diaminoderivative (XV) in which the substituent at position 2 is H, alkyl, SMe, or any group other than those (e.g. OH, NH_2, SH) capable of prototropic change, for it will



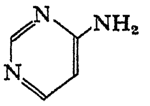
XI*a*



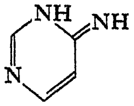
XI*b*



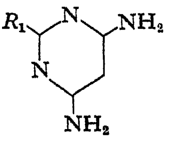
XIII



XIV



XIV*a*



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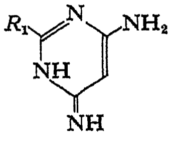
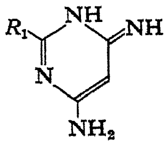


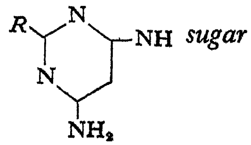
XV*a*



XV*b*

be seen that in such a compound the tautomeric possibilities (XV *a* and XV *b*) are limited so that in each form one of the two amino groups is unaffected and should show to some extent true amino character. This was found to be the case. In boiling ethyl alcohol under conditions allowing water formed in the reaction to be removed continuously, 4:6-diaminopyrimidine derivatives of the type discussed condense in presence of acidic catalysts (ammonium chloride, hydrogen chloride) giving crystalline glycosides (Baddiley, Lythgoe & Todd, 1943 *b*). Some representatives of this class so far obtained are listed in Table 1. They are all very readily hydrolysed by dilute mineral acids.

Table 1. *4-Glycosidaminopyrimidine derivatives*

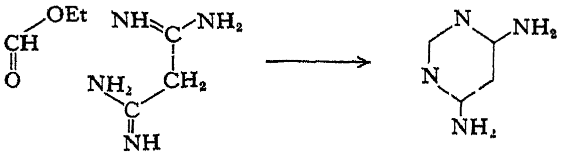


Sugar	Aglycone	m.p., $[\alpha]_D$
d-Xylose	4:6-Diaminopyrimidine	(*a*) m.p. 207° (d.); $[\alpha]_D = +154°$ (water) (*b*) Syrup
d-Xylose	4:6-Diamino-2-methyl-pyrimidine	m.p. 219° (d.); $[\alpha]_D = +158°$ (water)
d-Xylose	4:6-Diamino-2-methyl-thiopyrimidine	(*a*) m.p. 190–192° (d.); $[\alpha]_D = -20°$ (water) (*b*) Syrup
d-Glucose	4:6-Diaminopyrimidine	m.p. 209° (d.)
d-Glucose	4:6-Diamino-2-methyl-thiopyrimidine	m.p. 150° (d.); $[\alpha]_D = +112°$ (water)
d-Glucose	4:6-Diamino-2-dimethyl-aminopyrimidine	Syrup
l-Arabinose	4:6-Diamino-2-methyl-thiopyrimidine	Syrup
d-Ribose	4:6-Diaminopyrimidine	m.p. 158° (d.); $[\alpha]_D = -32°$ (water)
d-Ribose	4:6-Diamino-2-methyl-thiopyrimidine	m.p. 138–140° (d.)
d-Mannose	4:6-Diamino-2-methyl-thiopyrimidine	m.p. 213–214° (d.)
d-Galactose	4:6-Diamino-2-methyl-thiopyrimidine	m.p. 176° (d.); $[\alpha]_D = -58°$ (water)

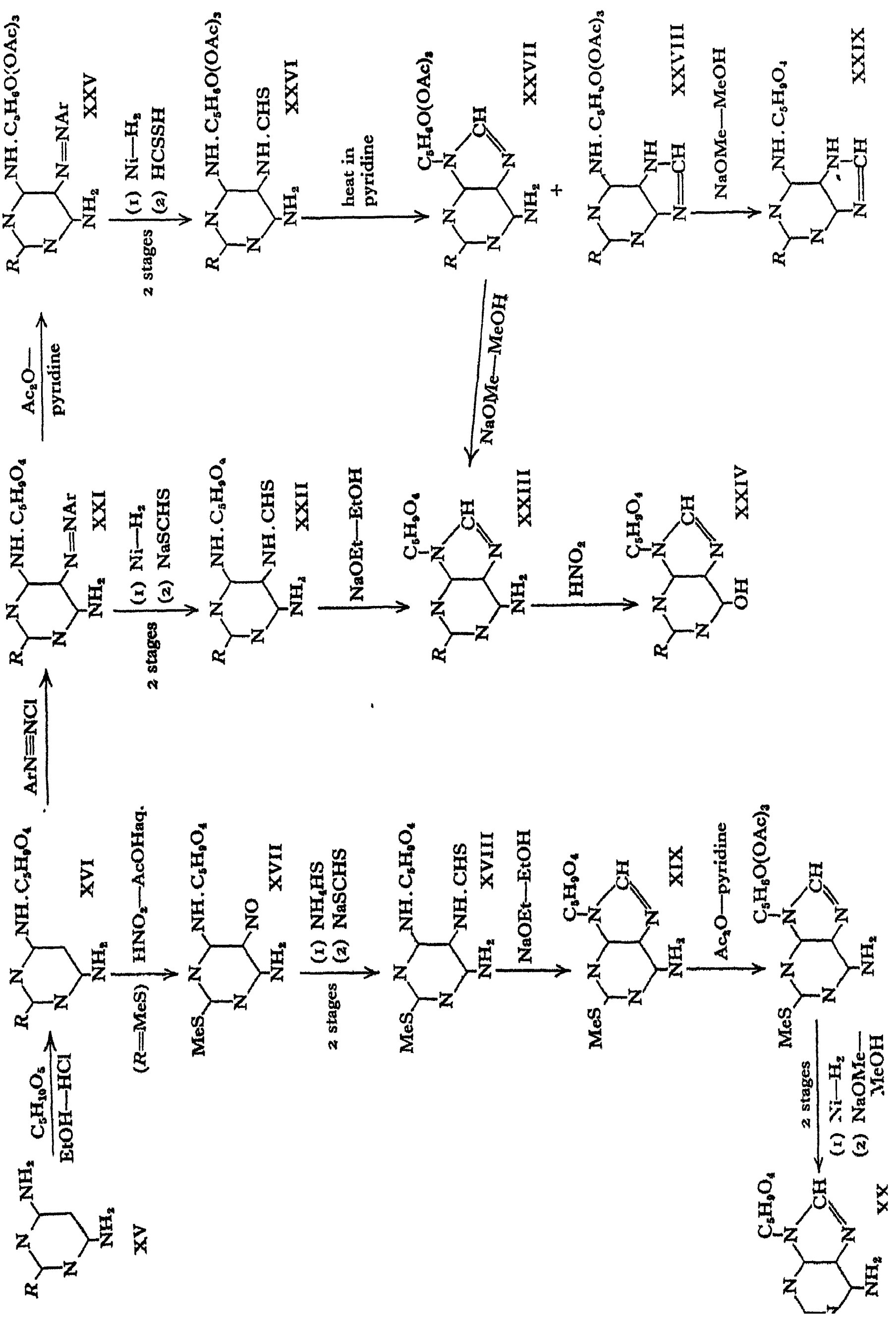
We were now concerned with methods for converting the synthetic glycosides into the corresponding 5-amino derivatives. Sugar-free 4:6-diaminopyrimidine derivatives can be converted into the corresponding 5-nitroso derivatives by nitrous acid in dilute acetic acid, and as these are readily reduced to 5-aminopyrimidines we at first intended to use similar methods on our glycosidaminopyrimidine derivatives. This method is in fact practicable where the substituent at position 2 is a methylthio group, and frequently offers the most convenient route to 5-amino-4-glycosidaminopyrimidine derivatives of this class. However, where other substituents less active in increasing the electron density over the nucleus are present at position 2, introduction of a nitroso group at C_5 requires the presence of mineral acids, which lead to hydrolysis of the glycosidic linkage, so that alternative procedures were found necessary. A more general method was found in the reaction of the glycosides with reactive diazonium compounds (that from 2:5-dichloroaniline is convenient). In weakly alkaline solution coupling takes place at C_5 in the pyrimidine nucleus to give crystalline arylazo compounds. The latter can be split reductively by catalytic or chemical methods to give the desired 5-aminopyrimidine derivatives. These were then cyclized to the corresponding purines. Before setting out schematically the way in which the synthetic methods so established have been applied to the first unambiguous syntheses of 9-glycosidoadenine derivatives, two points remain to be considered.

The first concerns the use of acetylated 5-thioformamidopyrimidine derivatives (XXVI) for the final cyclization procedure. The acetylated compounds (XXVI) can be obtained either by acetylation of the acetyl-free derivatives (XXII), or in better yields by carrying out acetylation at the arylazoglycoside stage (XXI) to give the acetylazo compound (XXV), which is then reduced and thioformylated. In our early experiments where anhydrous pyridine was used as the cyclization medium, protection of the hydroxyl groups of the sugar residue in this way was found desirable for the sake of good yields. It was found in certain cases that two acetylated glycosidopurines were then formed in the cyclization, (XXVII) and (XXVIII), giving on deacetylation the desired 9-glycosidoadenine derivative (XXIII) and the 6-glycosidaminopurine derivative (XXIX) respectively (Baddiley, Lythgoe & Todd, 1944). There is no difficulty in distinguishing the two glycosides, since (XXIX) contains a replaceable hydrogen atom in the iminazole ring and is readily soluble in dilute alkali, whereas (XXIII) is not. Conclusive proof that the structures assigned to these glycosides are correct has been obtained; for example, (XXIX) has been converted into the 9-methyl derivative from which on hydrolysis the authentic 9-methyladenine derivative was obtained. The 9-glycosidoadenine derivatives (XXIII), unlike the isomeric compounds, can be deaminated to give the 9-glycosidohypoxanthine derivatives (XXIV). Cyclization experiments with acetyl-free thioformamido compounds do not, so far as we have observed, give rise to the 6-glycosidaminopurine derivatives, but only to the 9-glycosidoadenine derivatives. In our more recent work a solution of sodium methoxide in ethyl alcohol has been employed for cyclization purposes (Kenner & Todd, 1946), and when applied to acetylated thioformamido compounds (XXVI) gives only 9-glycosidoadenine derivatives; here deacetylation undoubtedly occurs prior to cyclization. Whereas, therefore, cyclization of the acetyl-free thioformamido compounds proceeds in the way suggested by the model experiments on synthesis of 9-alkyladenine derivatives mentioned earlier, the acetylated compounds behave abnormally, and some explanation of this behaviour is required. We believe that the phenomenon in question is part of a more general abnormality existing in fully acetylated sugar derivatives, for which the most reasonable explanation is the interference at the glycosidic centre (or the groups attached to it), by the acetyl residue at C_2 of the sugar ring. In this case, for example, it seems likely that a chelate ring is formed between this residue and the glycosidic NH grouping (Kenner, Lythgoe & Todd, 1944). This would have the effect of increasing electron availability at the glycosidic nitrogen atom, and indirectly of that of the NH_2 group at position 6 in the pyrimidine ring, so enabling the latter to take part in the cyclization process. A chelate ring of this kind (7-membered) is sterically possible, and is supported by the behaviour of acetylated glycosidaminopyrimidines in quite a different connexion which will be referred to later.

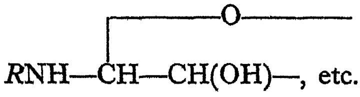
A second point deserving comment is the use of 2-methylthio derivatives as intermediates in the synthesis of 9-glycosides of adenine. For direct synthesis of such compounds the starting material required is 4:6-diaminopyrimidine itself (XV; $R=H$). No satisfactory method of obtaining this in quantity was available, and its use was only made possible by the discovery of a novel method (Kenner, Lythgoe, Todd & Topham, 1943; Howard, Lythgoe & Todd, 1944) for the formation of the pyrimidine ring, in which carboxylic esters are condensed with malondiamidine, e.g.

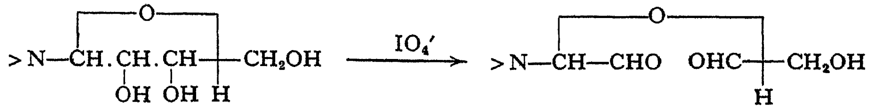


Whilst in some glycosidizations this derivative can be used satisfactorily, it is in general far less reactive than analogues containing an activating substituent at position 2; of these, 4:6-diamino-2-methylthiopyrimidine (XV; $R=MeS$) is readily accessible and the glycosides obtained from it (XVI; $R=MeS$) can frequently be converted to the 5-thioformamido derivatives (XVIII) by the nitrosation procedure. The 9-glycosido-2-methylthioadenines (XIX) obtained by ring closure can be acetylated and desulphurized by the use of the method of Mozingo, so giving ready access to the 9-glycosides of adenine itself (Howard, Lythgoe & Todd, 1945). These applications of the synthetic methods described are set out schematically opposite:

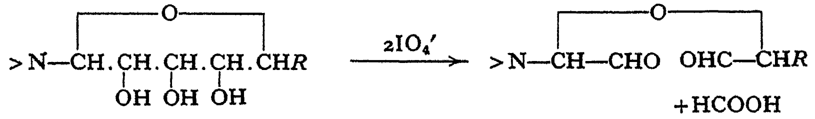

XV
C5H10O5
EtOH—HCl
XVI
ArN═NCl
XXI
Ac2O—pyridine
XXV
(R═MeS)
HNO2—AcOHaq.
XVII
2 stages
(1) Ni—H2
(2) NaSCHS
XXII
(1) Ni—H2
(2) HCSSH
XXVI
(1) NH4HS
(2) NaSCHS
XVIII
NaOEt—EtOH
XXIII
heat in pyridine
XXVII
+
XXVIII
NaOMe—MeOH
XXIX
XIX
HNO2
XXIV
Ac2O—pyridine
(1) Ni—H2
(2) NaOMe—MeOH
XX

Among the 9-glycosidoadenine derivatives so far obtained by these methods are the 9-*d*-xylosides of adenine (Kenner, Lythgoe & Todd, 1944), 2-methyladenine (Baddiley, Lythgoe & Todd, 1944), 2-methylthioadenine (Howard, Lythgoe & Todd, 1945) and the 9-*d*-riboside (Baddiley, Kenner, Lythgoe & Todd, 1944), 9-*d*-glucoside (Holland, Lythgoe & Todd, unpublished) and 9-*d*-mannosides (Lythgoe, Smith & Todd, 1946) of adenine. Of these, most interest attaches to 9-*d*-ribosidoadenine, prepared in the hope that the compound obtained would be identical with adenosine. Although the properties of natural and synthetic ribosides were very similar the two compounds proved to be isomers. The structural features of our synthetic compound are established by the method of synthesis, except in so far as concerns the size of the lactol ring and the nature of the configuration at the glycosidic centre; those of the natural nucleoside in all respects except the α, β nature; the possibility that its formulation as a 9-riboside from the spectral evidence might be erroneous was entertained, but considered unlikely, as our own authentic 9-*d*-ribosidoadenine showed almost identical ultra-violet absorption. In order to determine along what lines to modify our synthetic route so as to allow preparation of adenosine these different points were examined in turn.

It seemed that the most probable cause of the isomerism between adenosine and our synthetic glycoside was the size of the lactol-ring system in the latter, so that as a first step methods of determining ring size were sought. The classical procedure of methylation and hydrolysis which had served for the natural nucleosides required too much material to be suitable for our purpose, and the periodate oxidation method developed for use in the O-glycoside series by Hudson and collaborators (Jackson & Hudson, 1937; Jackson & Hudson, 1939; Richtmyer & Hudson, 1943) seemed to be more attractive, provided it could be established that N-glycosides behaved towards this reagent in the same way. Preliminary experiments on our glycosidaminopyrimidine derivatives showed that they were broken down completely by the reagent so that their structure cannot be determined in this way. Later work (Howard, Kenner, Lythgoe & Todd, 1946) has convinced us that in secondary N-glycosides  $R\mathrm{NH{-}CH{-}CH(OH){-}}$ (with O bridging from CH), etc., the iminoglycol structure at C_1-C_2 is oxidized in the same way as an α-glycol grouping, which then causes oxidation of the entire sugar system. If this interpretation is true, tertiary N-glycosides should be free from this difficulty and should oxidize by a route analogous to that obtaining in the O-glycosides. This was tested (Lythgoe & Todd, 1944) by model experiments in which suitable furanosides were found in the natural purine nucleosides, and pyranoside members of known structure were provided by the readily accessible theophylline-7-pentosides and -hexosides obtained by the method of Fischer & Helferich (1914) referred to earlier. These models behaved in the manner expected, a pentofuranoside requiring 1 mol. oxidant, whilst pentopyranosides (XXX; $R = \mathrm{H}$) and hexopyranosides (XXX; $R = \mathrm{CH_2OH}$) consumed 2 mol. oxidant and liberated 1 mol. formic acid:



$$>\mathrm{N{-}CH.\underset{OH}{CH}.\underset{OH}{CH}{-}\underset{H}{|}{-}CH_2OH} \xrightarrow{\mathrm{IO_4'}} >\mathrm{N{-}CH{-}CHO\quad OHC{-}\underset{H}{|}{-}CH_2OH}$$

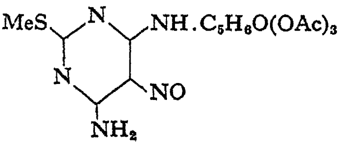


$$>\mathrm{N{-}CH.\underset{OH}{CH}.\underset{OH}{CH}.\underset{OH}{CH}.CH}R \xrightarrow{\mathrm{2IO_4'}} >\mathrm{N{-}CH{-}CHO\quad OHC{-}CH}R \; + \mathrm{HCOOH}$$

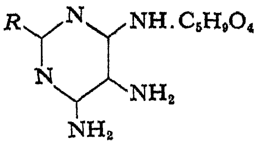
XXX

so that titrimetric investigation of the ring structure of 9-glycosidoadenine derivatives is possible with quite small quantities of material. Adenine riboside, in common with all the other glycosides obtained by the synthetic route described above, was found to consume 2 mol. of metaperiodate and to liberate 1 mol. formic acid. They are therefore pyranosides.

Clearly this fact alone is sufficient to explain the isomerism between the synthetic compound and adenosine (adenine-9-*d*-ribofuranoside), but it will be convenient to discuss at this stage the steps taken to exclude the other possibilities. The first of these concerns the α, β nature of the natural and synthetic glycosides. Earlier work (Kenner, Lythgoe & Todd, 1944; Howard, Lythgoe & Todd, 1945) had shown that glycosidization of 4:6-diaminopyrimidine derivatives (e.g. with *d*-xylose) may in some cases lead to formation of two isomeric compounds, e.g. (XVI; $R = \mathrm{MeS}$, $\mathrm{C_5H_9O_4} = d$-xylosyl). These gave rise to two series of isomeric derivatives, e.g. the 5-nitroso derivatives (XVII; $\mathrm{C_5H_9O_4} = d$-xylosyl) and 5-arylazo derivatives (XXI; $R = \mathrm{MeS}$, $\mathrm{C_5H_9O_4} = d$-xylosyl), and it was hoped that by using these as intermediates in the synthetic method it would be possible to obtain isomeric 9-glycosidopurine derivatives. When this was attempted, both series gave rise to one and the same 9-glycosidoadenine derivative; in the example cited (XIX; $\mathrm{C_5H_9O_4} = d$-xylosyl). It was then found that the loss of isomerism took place when the 5-nitroso or 5-arylazo derivatives were reduced to give the corresponding 5-amino derivative. Similar loss of isomerism also occurs when the isomeric 5-nitroso derivatives (XVII; $\mathrm{C_5H_9O_4} = d$-xylosyl) are acetylated; from each isomer the same triacetylnitroso derivative (XXXI) is obtained. It was soon established that the isomerism between the two series of derivatives was due to differences in the sugar component, but the exact nature of the differences, and why interconversion should occur during reduction or acetylation reactions, was for some time obscure. It will not be possible here to do more than summarize the results of our investigations on this subject (Howard, Kenner, Lythgoe & Todd, 1946), but we believe that they have led to a satisfactory understanding of the phenomena described, which are now seen to fit in well with the general behaviour of N-glycosides. The facility with which α, β changes occur in such compounds has been known for many years, and it has now been shown that furanoside$\rightleftharpoons$pyranoside changes can also occur under conditions where ordinary O-glycosides would be quite stable. Whilst it is not possible to determine the ring structures of the isomeric glycosidaminopyrimidine derivatives directly by the periodate oxidation method, consideration of the available evidence shows that they are all pyranosides, and that the loss of isomerism during the synthesis



XXXI

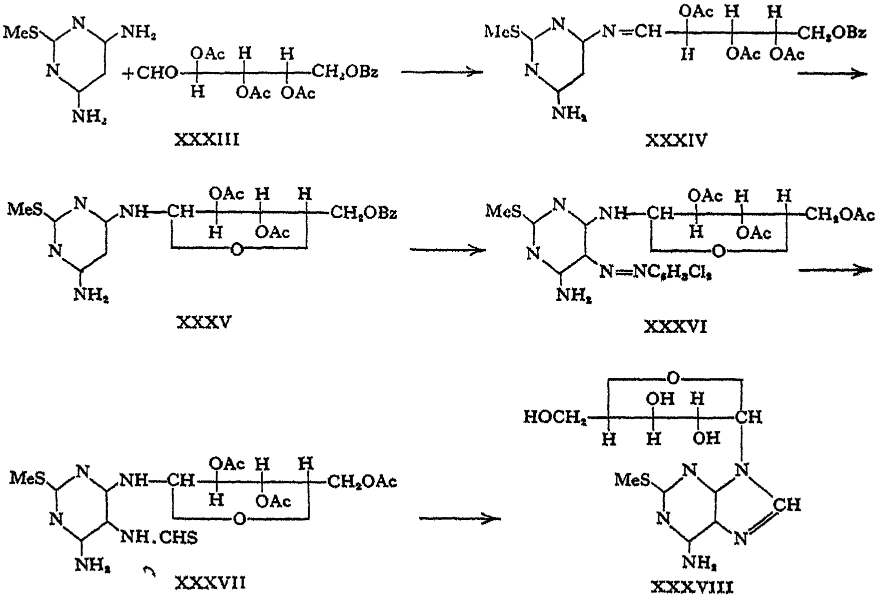


XXXII

does not involve a change of lactol-ring structure, as it can take place in fully acetylated structures such as the isomeric compounds (XXVI; R = H, $C_5H_9O_4$ = *d*-xylosyl) where such changes are excluded. The isomerism must therefore be of the α, β type, and it is perhaps not surprising that glycosidization under our conditions should give rise to α, β-pyranoside mixtures, since they are closely similar to those under which α- and β-methylglucopyranosides are formed in E. Fischer's method for O-glycosides. The α, β interconversion reactions which must take place when isomerism is lost in the above changes, and which appear rather remarkable at first sight, are now regarded as examples of mutarotational change. It is shown that in those derivatives capable of existing in two isomeric forms (e.g. XVI, XVII; XXI) structural features promoting ease of mutarotational change are absent, whilst in those existing in only one form (e.g. XXXI, XXXII) such features are present, but because one of the members of the α, β pair is considerably more stable than the other, only this member is isolated from each of the α, β forms of the starting material. The most important factor controlling the ease of mutarotational change appears to be the electron availability at the glycosidic nitrogen atom. This explains why loss of isomerism occurs on acetylation or reduction of the isomeric forms of (XVII, $C_5H_9O_4 = d$-xylosyl). In the former case the previously mentioned chelation between the glycosidic NH group and the acetyl group at C_2 in the sugar ring is responsible for the increased basicity; in the latter case the increase is caused by the introduction of the strongly basic 5-amino group; in either case the effect is to facilitate mutarotational change so that only one form of the product is isolated. In all cases so far examined it has been found that it is the β form of the 5-amino derivative (XXXII) which is the more stable. In these compounds the configuration can be deduced from measurements of optical rotation (Howard, Kenner, Lythgoe & Todd, 1946). It is almost certain therefore that the 9-glycosidopurines obtained from them are β-glycosides. This has in fact been shown to be the case by chemical methods (Davoll, Lythgoe & Todd, 1946), and since the same methods are applicable to the natural purine nucleosides they will be dealt with at this point.

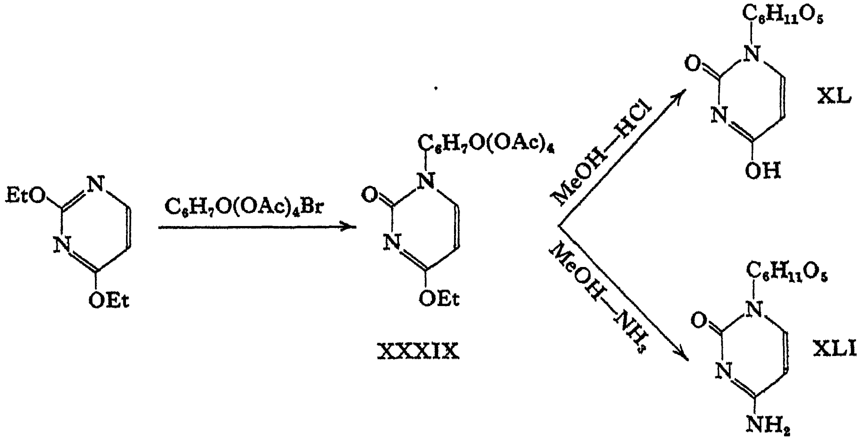
It is usually assumed that in Fischer & Helferich's synthesis of purine glycosides, replacement of a halogen atom of the acetohalogeno sugar with a purine residue is accompanied by Walden inversion, so that, for example, from α-acetobromoglucose, β-glucosides are formed. It seemed possible to use this as a basis for the determination of configuration in purine glycosides, provided that its correctness could be established. Our most recent results indicate that the assumption is fully warranted in the case of the *cis*-acetohalogeno sugars such as those derived from glucose, galactose, xylose and arabinose, but not in the case of the *trans*-acetohalogeno sugars from ribose, rhamnose, mannose, etc. The latter appear to behave abnormally in the Fischer-Helferich procedure, as indeed they do in other reactions, but this need not be considered further here, since for our purposes only *cis*-acetohalogeno sugars need be used. Acetochloroxylose is a *cis*-α-halogenose, so when reacted with purine derivatives it should give rise to β-xylosides. The adenine β-xylopyranoside obtained by dehalogenating and deacetylating the product of reaction of acetochloroxylose and 2:8-dichloroadenine silver was found to be identical with the 9-*d*-xylopyranosidoadenine prepared by our synthetic method; the latter is therefore a β-xyloside, and this agreement with the results obtained from optical data strengthens the plausibility of the original assumption. The adenine glucoside obtained by Fischer & Helferich using *cis*-α-acetobromoglucose is therefore almost certainly a β-glucoside. On oxidation with periodate this compound takes up 2 mol. of oxidant giving a dialdehyde. Since the configuration at C_4 in *d*-ribose is identical with that at C_5 in *d*-glucose, and since in the oxidation asymmetry at all centres other than C_1 is destroyed, then if adenosine is a β-glucoside both adenine glucoside and adenosine should give on oxidation the same dialdehyde. This was found to be the case, and adenosine must be a β-glycoside. The same dialdehyde also arises from periodate oxidation of the adenine-9-*d*-mannopyranoside obtained by our synthetic method (Lythgoe, Smith & Todd, 1946). This shows that the latter is a β-glycoside, but a further conclusion follows. Since our synthetic method can give rise only to N_9- and never to N_7-glycosides the sugar residue in adenine glucoside and adenosine must also be located at N_9 thus providing unambiguous confirmation of the results obtained from the spectroscopic evidence.

These points being established a synthesis of adenosine seemed possible if the initial glycosidisation could be modified so that a glycofuranose residue could be introduced into the amino group of a suitable 4:6-diamino-pyrimidine derivative. Early experiments in which this was attempted by use of 5-substituted glycofuranose derivatives (e.g. 5-benzoyl-*d*-ribofuranose) proved disappointing, and although use of acetylated furanose sugars in which C_1 was unprotected (e.g. 2:3:5:6-tetra-acetyl-*d*-galactofuranose) gave more promising results the best method obtained so far is an unusual glycosidization procedure employing open chain sugars as intermediates (Kenner, Lythgoe & Todd, 1946). This was first worked out using *l*-arabinose as model sugar on account of its comparatively ready accessibility. 5-Benzoyl-2:3:4-triacetyl-*aldehydo*-*l*-arabinose (XXXIII) prepared by standard methods was condensed with 4:6-diamino-2-methylthiopyrimidine to give the Schiff's base (XXXIV) from which protecting acetyl groups were removed selectively by methanolic ammonia. In the absence



of protecting groups at possible points of lactol-ring closure Schiff's bases of this type have no more than a transient existence, undergoing rearrangement to the true N-glycoside. The protecting benzoyl group at C_5 ensures that this shall be of the furanoside type (XXXV). After removal of the benzoyl group, this compound was converted by diazo coupling and acetylation into the acetylated arylazo compound (XXXVI), which was reduced and thioformylated in the usual way to give (XXXVII) from which by cyclization with sodium methoxide in alcohol the 9-*l*-arabofuranosido-2-methylthioadenine (XXXVIII) was obtained. That it is a 9-glycoside is shown by its insolubility in alkali, and its furanoside nature has been demonstrated by periodate oxidation. Compounds of this type can, as mentioned above, be readily desulphurized, so that a similar method using *d*-ribose instead of *l*-arabinose derivatives may be expected to yield adenosine, since the two sugars probably behave similarly in the respects essential to this method.

Pyrimidine ribonucleosides. No synthesis of a naturally occurring member has so far been recorded, although Hilbert and coworkers (Hilbert & Johnson, 1930; Hilbert & Jansen, 1936; Hilbert, 1937) have described methods by which pyranose analogues can be obtained. The methods used are similar to those employed by Fischer & Helferich in the purine series, but since the natural nucleosides are definitely known to be N_3-glycosides, the ambiguity concerning the position of the sugar residue in products synthesized in this way seems less important. Hilbert's method is shown below:



XXXIX

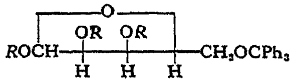
XL

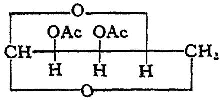
XLI

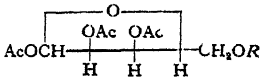
2:6-Diethoxypyrimidine and acetobromoglucose react to give a tetra-acetyl glucoside (XXXIX), from which protecting acetyl and ethyl residues are removed either by methanolic hydrogen chloride to give uracil-3-*d*-glucoside (XL) or by methanolic ammonia to give cytosine-3-*d*-glucoside

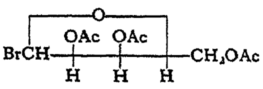
(XLI). The method has been applied to acetobromoribopyranose to give a uracil 3-*d*-riboside (Hilbert & Rist, 1937) isomeric with natural uridine from which it certainly differs in ring structure, and possibly also in the configuration at the glycosidic centre; this point has not yet been investigated.

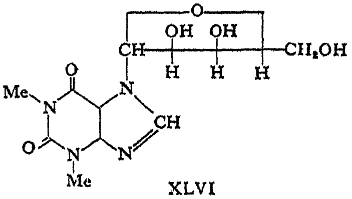
By use of periodate oxidation we have obtained the same dialdehyde from uridine as from the uracil glucoside mentioned above, and a common dialdehyde from cytidine and cytosine glucoside (Davoll, Lythgoe & Todd, 1946). The method of synthesis of the glucosides shows that they are *β*-compounds, so that a similar structure is present in the natural nucleosides. It also follows that the synthetic compounds are N_3-glucosides; formerly they had been allotted this structure largely on the grounds of analogy. Consequently, it seemed feasible to use this method for the synthesis of the natural nucleosides provided a method for the preparation of acetobromoribofuranose could be found. Bredereck, Köthnig & Berger (1940) attempted to obtain this compound by using as intermediate 5-trityl-*d*-ribose (XLII, *R* = H). The 5-trityl-1:2:3-triacetyl-*d*-ribose (XLII, *R* = Ac) obtained by acetylation was treated with hydrogen bromide in acetic acid, but simultaneous removal of trityl residue and of the acetyl group at C_1 took place, giving 2:3-diacetyl-anhydroribose ⟨1:5⟩ ⟨1:4⟩ (XLIII). We have overcome this difficulty by removing the trityl group by hydrogenation (Howard, Lythgoe & Todd, 1946). The resulting 1:2:3-triacetylribofuranose (XLIV, *R* = H) gives on acetylation a crystalline 1:2:3:5-tetra-acetylribofuranose (XLIV, *R* = Ac) which can be converted by liquid hydrogen bromide into the desired acetobromofuranose (XLV). Although the latter has not yet been obtained crystalline there is little doubt of its identity, since by reaction with theophylline silver followed by deacetylation, it gives a theophylline 7-*d*-ribofuranoside (XLVI), the structure of which has been established by periodate oxidation. Subsequent work has shown that it is possible to use this acetobromoribofuranose successfully for the preparation


XLII


XLIII


XLIV


XLV

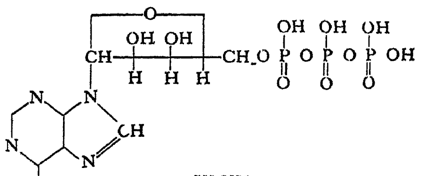

XLVI

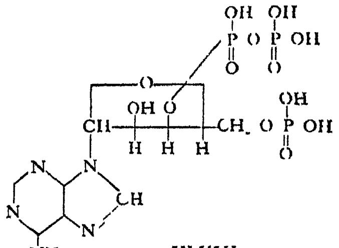
of pyrimidine ribofuranosides. By condensation of (XLV) and 2:6-diethoxypyrimidine followed by removal of protecting residues from the product with methanolic ammonia we have obtained a *d*-ribofuranosidocytosine identical with natural cytidine.

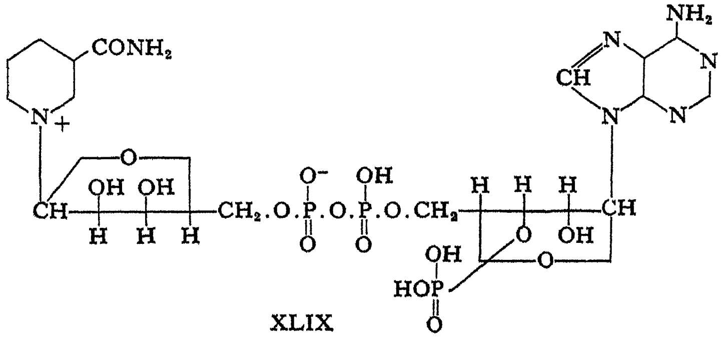
II. NUCLEOTIDES

The structural question arising in these compounds is the location of the phosphate residue(s). Conclusive evidence on this point is available for the nucleotides derived from breakdown of nucleic acids only in the case of guanylic and yeast adenylic acids. The *d*-ribose phosphate obtained by acid hydrolysis of the deamination products, xanthylic and inosine phosphoric acids was found by Levene & Harris (1932) to give on reduction an optically inactive 3-phosphoribitol. The phosphate residue in these nucleotides therefore esterifies the hydroxyl at $C_{3'}$. The evidence available in the case of the pyrimidine ribonucleotides is sufficient to show that one of the hydroxyl groups present at $C_{2'}$ and $C_{3'}$ is esterified by a phosphate residue; most convincing is the demonstration that uridine-5′-phosphate, obtained from uridine by unambiguous partial syntheses (Levene & Tipson, 1934*b*; Bredereck & Berger, 1940), is different from natural uridylic acid. Position $C_{3'}$ is preferred by analogy with the purine analogues. In the deoxyribonucleotides similar grounds lead to an analogous formulation, but experimental confirmation is lacking. Clarification of these points by synthetic methods would be desirable. In muscle adenylic acid the phosphate group undoubtedly esterifies the $C_{5'}$ hydroxyl group, as shown by both degradative (Levene & Mori, 1929) and synthetic work (Levene & Tipson, 1937).

The adenine nucleotide coenzymes all contain the muscle adenylic acid residue, but in the polyphosphate members some uncertainty exists regarding the location of some of the phosphate groups. For example, adenosine triphosphate is built up from a molecule of muscle adenylic acid and a pyrophosphoryl group, and on the basis of titration data, which revealed the presence of three primary and one secondary dissociations, was allotted structure (XLVII) by Lohmann (1932, 1935). Other workers have, however, claimed that it is possible by use of certain enzyme preparations to remove the acid-stable (5′) phosphoryl group from adenosine triphosphate, leaving the acid-labile residues (those of the pyrophosphoryl group) intact; this suggested that the latter group is attached to one of the hydroxyls of the ribofuranose residue (structure XLVIII). Since muscle adenylic acid has been shown (Lythgoe & Todd, 1944) to consume 1 mol. periodate, whilst yeast adenylic acid, in which one of the two α-glycollic hydroxyls required for oxidation is protected by a phosphoryl residue, is inert to this reagent, it should be possible to decide between these alternative formulae. It has now been found (Lythgoe & Todd, 1945) that adenosine triphosphate is oxidized by sodium metaperiodate with uptake of 1 mol. oxidant. This we regard as conclusive proof of Lohmann's formula (XLVII). Adenosine diphosphate is therefore to be regarded as adenosine-5′-pyrophosphate, which further suggests that cozymase, from which the pyrophosphate can be obtained by alkaline cleavage, is correctly represented by (II). Given a pure sample of triphosphopyridine nucleotide it would appear to be a simple matter to determine in the same way whether the adenosine and nicotinamide nucleoside residues are united through a triphosphate linkage, as early formulations indicated, or whether, as suggested by Schlenk (1943), the third phosphoryl group esterifies the adenylic acid residue at $C_{3'}$ as in (XLIX).


XLVII

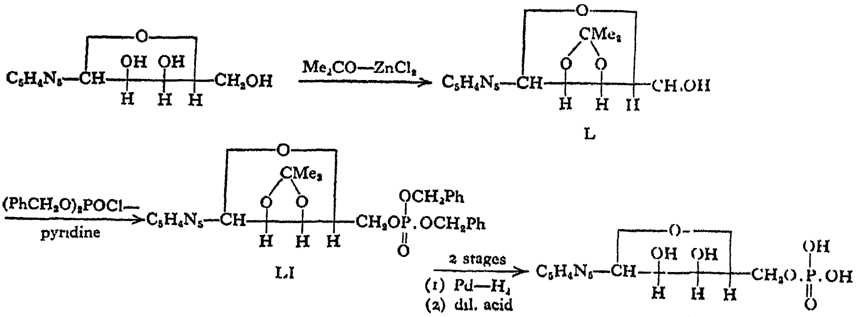

XLVIII


XLIX

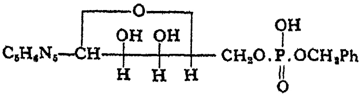
Synthesis. Investigations concerned with partial synthesis of nucleotides have been undertaken by a number of workers. Differences of approach are here confined mainly to (*a*) the nature of the phosphorylating agent used and (*b*) the methods employed to direct the entering phosphoryl group to the desired position in the molecule. Earlier workers generally made use of phosphoryl chloride as phosphorylating agent (Fischer, 1914), but more recently other phosphorylating agents have been described including diphenyl chlorophosphonate (Brigl & Müller, 1939), phenyl dichlorophosphinate (Brigl & Müller, 1939; Gulland & Hobday, 1940) and catechol oxychlorophosphine (Reich, 1946), some of which have been employed in the nucleotide field. Without preliminary protection of the hydroxyl groups at C_2 and $C_{3'}$ Jachimowicz (1937) converted adenosine to muscle adenylic acid by phosphoryl chloride in pyridine, and Gulland and collaborators (Gulland & Hobday, 1940; Barker & Gulland, 1942) have shown that guanosine, adenosine and uridine can be converted into the corresponding natural nucleotides by using barium hydroxide solution in place of pyridine as base.

Partial and complete protection of hydroxyl groups whose esterification is not desired offers the advantage of increasing the yield, and of proving with lesser or greater degree of certainty the structure of the products so obtained. Trityl uridine and trityl cytidine have been converted to the nucleotides uridylic (Bredereck & Berger, 1940) and cytidylic (Bredereck, Berger & Ehrenberg, 1940) acids by phosphorylation (using diphenyl chlorophosphonate) followed by removal of protecting groups. The unambiguous syntheses of inosinic acid (Levene & Tipson, 1935) and muscle adenylic acid (Bredereck, Berger & Ehrenberg, 1940; Levene & Tipson, 1937), carried out by Levene and his collaborators using phosphoryl chloride and pyridine as reagent, and by Bredereck and his collaborators using diphenyl chlorophosphonate, use as starting materials the $C_{2'}$:$C_{3'}$-monoacetone derivative or the 2′:3′-diacetyl derivative of the appropriate nucleoside. No 2′-phosphonucleoside has so far been obtained synthetically. This is the more to be regretted since examination of the stability of such compounds to hydrolytic agents is desirable in order to elucidate why neither such compounds nor the corresponding 5′-phosphonucleosides are obtained on hydrolysis of polynucleotides.

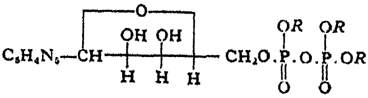
The yields obtained in the syntheses referred to above are very low and preclude their use for preparative purposes. One of us (A. R. T.) has recently commenced an extended study of methods for phosphorylation of sensitive molecules, in a search for procedures which may be flexible enough to be capable of extension to polyphosphoric esters. It would be premature at this stage to attempt a survey of methods offering promise of fulfilling the necessary requirements, but reference may be made to one reagent to which some attention has been devoted, viz. dibenzylchlorophosphonate (Atherton, Openshaw & Todd, 1945). This substance obtained by action of chlorine on dibenzyl phosphite in an inert solvent, or by treatment of dibenzyl phosphate with thionyl chloride (Deutsch & Fernö, 1945), reacts readily with alcohols at room temperature in the presence of tertiary bases, to give esters from which protecting benzyl groups can be removed by catalytic hydrogenation. This method has been applied to synthesis of muscle adenylic acid (LII) by the route shown below (Baddiley & Todd, 1946) which furnishes the product in good yield. From dibenzyl phosphoryl esters of type (LI) it is frequently possible to remove one of the protecting benzyl residues catalytically or by other means, and indeed (LI) can be converted by action of dilute acid to the ester (LIII) which forms a monosilver salt. Since it has been demonstrated in model experiments that, for example, dialkyl or diaryl chlorophosphonates react with silver dibenzyl phosphate to yield pyrophosphates, it is evident that we have here a potential



LII



LIII



LIV

method for the synthesis of polyphosphates and for the linkage of different molecules through polyphosphate residues. Work in this field, in which technical difficulties are considerable, is still in progress. Already, however, it has been found that the monosilver salt of (LIII) can be condensed with dibenzylchlorophosphonate to give an ester (LIV, $R=CH_2Ph$) from which the benzyl groups can be removed by hydrogenolysis yielding an adenosine-5′-pyrophosphate (LIV, $R=H$) identical with natural adenosine diphosphate.

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THE BIOLOGICAL SYNTHESIS OF PURINE COMPOUNDS

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I. THE SEARCH FOR SPECIFIC MICRO-PURINE METHODS

The renewed interest in the group of substances which include purine and pyrimidine compounds and the nucleic acids, the main topic of this symposium, has quite naturally turned the spotlight on the enzymes of purine and pyrimidine metabolism as well as those of the nucleic acids. This particularly in the hope of finding the possible pathways by which the nitrogenous bases and their ribosides which constitute the building blocks of nucleotides and nucleic acids are synthesized, and to arrive at an understanding, on a chemical basis, of the biological action of the nucleic acids. The interest in the latter problem has been greatly intensified with the discovery of Avery, McLeod & McCarthy (1944) that the type transforming factor of pneumococci is a nucleic acid of the desoxyribose type.

A prerequisite for a deeper study of the mechanism of the enzymatic synthesis of this group of compounds was the development of micromethods which not only should satisfy the needs for high sensitivity, but in addition should fulfil the most rigid requirements for specificity. Otherwise, the methods would not be applicable, for instance, in a study of synthetic reactions taking place in biological systems such as crude tissue extracts. A large part of this report will be devoted to the description and evaluation of the type of specific micro-purine methods which have been developed for the above-mentioned purpose.

It is a well-known fact that purine compounds possess an intensive absorption in the ultra-violet region around 260 mμ. The molecular extinction coefficients (ϵ) of this group of compounds at their absorption maxima range from 12,000 to 15,000, intensities which must be considered high compared with those of other biological non-coloured structural substances such as amino-acids or carbohydrates. For further details concerning absolute ultra-violet spectroscopy the reader is referred to the fundamental studies of pure purines in the quartz spectrograph by Holiday (1930), by Heyroth & Loofbourow (1934) and by Gulland & Holiday (1936).

The absolute ultra-violet spectroscopy has been of much importance in biological research. Caspersson's (1940) introduction of chemical histology is a significant development in this direction. However, it goes without saying that absolute ultra-violet spectroscopy is not sufficiently specific to be used in a study of the mechanism of the metabolism or synthesis of purine compounds. This was also realized by several investigators in this field who, for instance, made use of the specific depolymerases in order to distinguish between ribonucleic acid and desoxyribonucleic acid (Gersh & Bodian, 1943; Brachet, 1941).

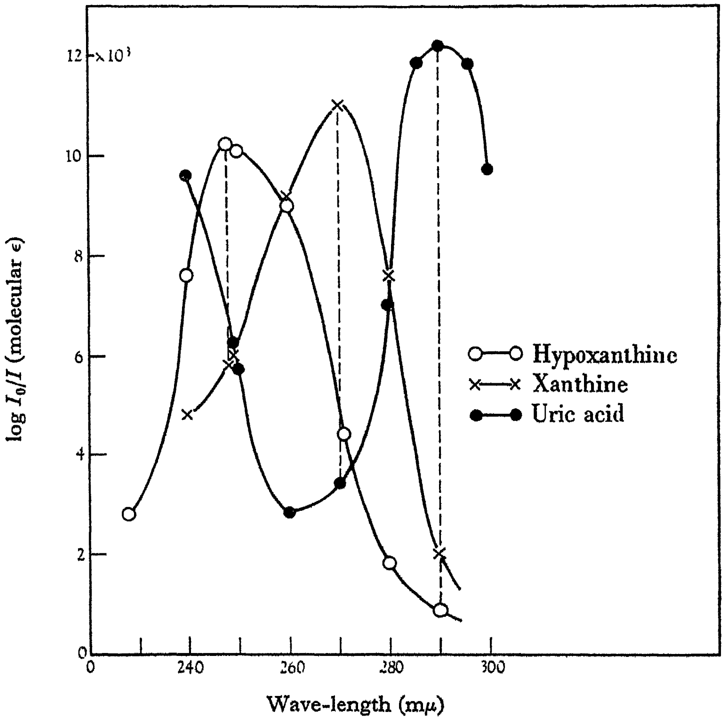

Fig. 1. Absorption curves in the ultra-violet of hypoxanthine, xanthine and uric acid. Abscissae: wave-length in mμ. Ordinates: absorption expressed as molecular extinction coefficient (ε).

II. DIFFERENTIAL ULTRA-VIOLET SPECTROSCOPY BY MEANS OF SPECIFIC ENZYMES

The application of specific enzymes of the purine system for analysis of this group of compounds was first introduced by Schmidt in 1928 and used by him and other investigators. One might in this connexion cite the interesting studies of Örström, Örström & Krebs (1939) on the formation of hypoxanthine from various precursors in liver slices.

Most of these specific enzymatic methods are unfortunately far from sensitive. It would, however, be feasible to develop sensitive as well as specific methods in all these cases in which specific enzymes of the purine metabolism by conversion of one purine compound to another caused marked alterations in that part of the ultra-violet spectrum in which the absorption intensity is high. The main question was, therefore, whether it is possible by oxidation and reduction or by deamination and reamination to alter the ultra-violet spectra of purines markedly. That such a possibility actually exists appears from Fig. 1 in which the ultra-violet spectra of three oxidation steps, hypoxanthine, xanthine and uric acid, are shown graphically. An analogous phenomenon is illustrated in Fig. 2, which shows the difference in ultra-violet spectra between adenine compounds and their deamination products, 6-hydroxy-purine compounds. If the proper enzymes can be isolated and purified the marked spectral changes, the Δε of which in several cases exceeds 10,000, will serve as a basis for a highly sensitive and specific method for the determination of various purine compounds.

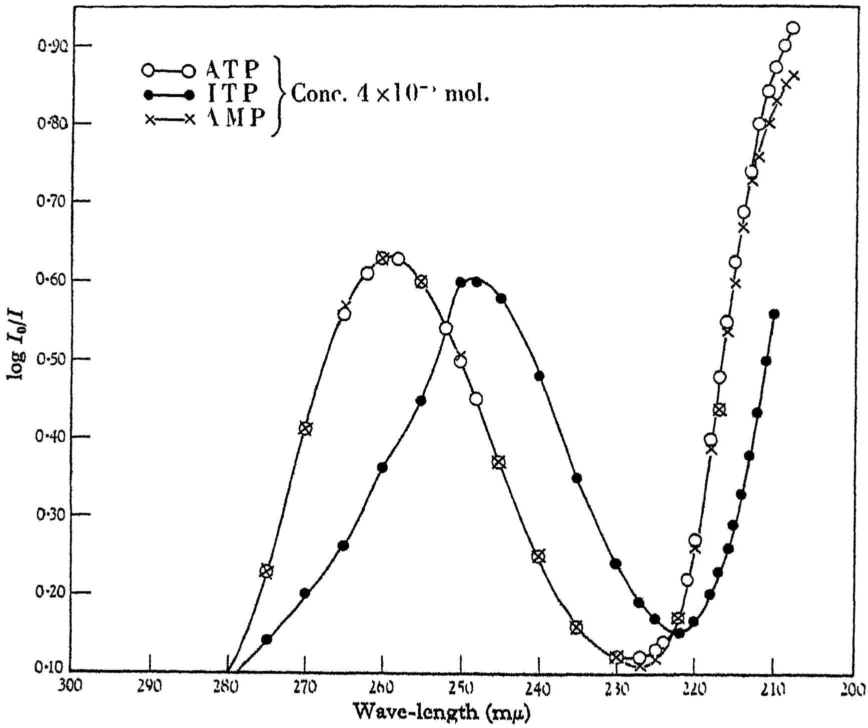

Fig. 2. Absorption curves in the ultra-violet of adenylic acid, adenosine triphosphate and inosine triphosphate. Abscissae: wave-length in mμ. Ordinates: absorption.

In Table 1 is listed the density changes ($\Delta \log (I_0/I)$) per μg. purine in 1 ml. for various conversions which purine compounds may undergo. The data are selected at the wave-lengths at which the density differences between the original compounds and the end-products are largest, both relatively and absolute. The importance of the practice of selecting more than one wave-length in the analyses will be discussed later.

Table 1. *Various transformations of purines*

Transformations	$\pm\Delta \log (I_0/I)$ for 1 μg. purine per ml.		
	290mμ	270mμ	248mμ
Hypoxanthine to uric acid	+0·080	—	−0·030
Xanthine to uric acid	+0·066	−0·053	—
Guanine to uric acid	+0·048	−0·030	—

Having established a basis for a differential ultra-violet spectroscopy the next step necessary was to obtain specific enzymes sufficiently active, and sufficiently freed from other enzymes, to bring about rapid oxidations or deaminations of the purine compounds to be investigated.

The purification of a number of enzymes of the purine metabolism was therefore undertaken. This included enzymes attacking the purine skeleton directly, such as oxidases and deaminases, as well as enzymes which attack other parts of the various purine nucleotides, such as nucleosidases, phosphatases and transphosphorylases. It would take too much space to describe the purification procedures involved. These procedures, as well as the studies of a number of enzymes of the purine metabolism, have been described in detail elsewhere (Kalckar, 1947*c*).

III. MICRO-DETERMINATION OF HYDROXY-PURINES

Hypoxanthine and xanthine were determined by measuring the increase in density at 290 mμ which developed upon addition of xanthine oxidase. It appears from Fig. 1 that the first oxidation step, i.e. hypoxanthine to xanthine, brings about a decrease at 248 mμ, whereas the second oxidation step, xanthine to uric acid, causes a marked decrease in density at 270 mμ. Since the xanthine oxidase prepared was free of guanase, the determination does not include guanine. Guanine can be estimated separately by addition of xanthine oxidase plus guanase. The method therefore permits a differentiation between xanthine and guanine. Uric acid was determined at 290 mμ by addition of uricase which brings about a marked decrease of the density at this wave-length.

The oxidases used are strictly specific for the free hydroxy-purine compounds. Thus, the ribosidic-bound hydroxy-purines do not react, until the ribose is split off either by acid hydrolysis at 100° or by enzymatic means, i.e. using nucleosidases.

Inosine was determined at 290 and at 248 mμ in the presence of nucleosidase plus inorganic phosphate which catalyse the liberation of hypoxanthine; the latter is instantaneously oxidized to uric acid by the presence of an excess of xanthine oxidase and oxygen. Under these conditions the fission of the riboside proceeds to completion.

The methods for the free as well as those for the ribosidic-bound hydroxy-purines give a satisfactory proportionality between concentration and density increase at 290 mμ (cf. Kalckar, 1946*a*).

IV. MICRO-DETERMINATION OF ADENOSINE COMPOUNDS

Adenine compounds are determined by measuring the decrease in density at 265 mμ and the increase in density at 240 mμ brought about by addition of specific deaminating enzymes such as adenosine deaminase or 5-adenylic acid deaminase.

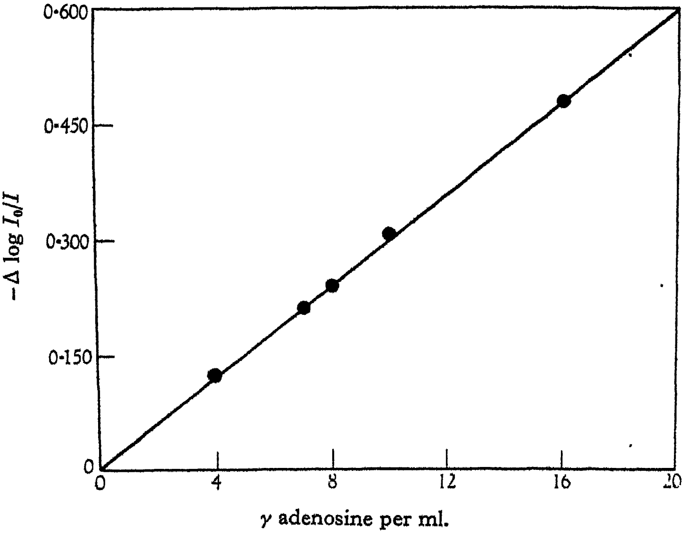

Fig. 3. The relation between adenosine concentration (μg. per ml.) and density change ($-\Delta \log (I_0/I)_{265}$) due to enzymatic deamination.

It appears from Fig. 3 that there is a good proportionality between the concentration of adenosine and the total decrease in density at 265 mμ caused by enzymatic deamination. A similar proportionality exists between adenylic acid concentration and the density decrease at 265 mμ.

Adenylpyrophosphate was determined at the same wave-lengths by adding a mixture of 5-adenylic acid deaminase and potato adenylpyrophosphatase. The results were always expressed as μg. adenyl radical per ml. One μg. adenyl radical per ml. corresponds to a $\Delta \log (I_0/I)_{265}$ of $-0{\cdot}019$ and a $\Delta \log (I_0/I)_{240}$ of $+0{\cdot}010$. Further technical details and data are published elsewhere (Kalckar, 1946*b*).

V. EVALUATION OF THE ENZYMATIC DIFFERENTIAL ULTRA-VIOLET SPECTROSCOPY

The main advantages of the methods described are the high sensitivity and the high specificity, quite apart from the moderate time required to perform the analyses which are usually finished within 30–40 min. The high sensitivity is due to the high extinction coefficients of purine compounds in the ultra-violet region between 250 and 300 mμ (ϵ about 12,000 or higher) and the marked differences in spectra. The high specificity is based on three features:

(1) The high specificity of the enzymes used in the analyses.

(2) The possibility of selecting appropriate wave-lengths. One wave-length at which the density increases, another at which it decreases, a third wave-length at which the density remains unaltered (isobestic point) during the enzymatic reaction. A definite quantitative relationship exists between the various density changes at the wave-lengths selected.

(3) The possibility of making a second enzymatic spectrophotometry on the end-product. An example of such a two-step analysis will be given.

It may be of interest to try to evaluate in a little more detail each of these three aspects.

The question of enzyme specificity resolves itself into the problem of obtaining enzyme preparations of sufficient purity and of testing such preparations on a variety of possible substrates. A few examples will illustrate this more clearly. Crude xanthine oxidase preparations contain among other things nucleosidase (Dixon & Lemberg, 1934), and would therefore be unsuitable as an analytical tool in a differentiation between free and ribosidic-bound purine. The purified xanthine oxidase is free of nucleosidase and can therefore be used in such an analysis. Occasionally the presence of one enzyme may conceal the presence of another enzyme. Such a complication was encountered in a study of embryonic rat livers. Fractions prepared in the same manner in which crude nucleosidase fractions from adult rat livers are prepared seemed to lack this enzyme. This was found to be due to interferences from small amounts of uricase which further oxidizes the uric acid formed in the nucleosidase test (splitting of hypoxanthine riboside plus oxidation of free hypoxanthine to uric acid at 290 mμ). The uricase was dispersed extremely finely and consequently not spun down at the speed (12,000–14,000 r.p.m.) which is sufficiently high to spin off the uricase particles in the extracts from the livers of adult rats. However, a clear separation between the two enzymes was obtained at a speed of 22,000 r.p.m., which makes it possible to spin off the finely dispersed uricase, leaving the nucleosidase in solution. It is possible to add many more examples than the two presented which illustrate the importance of testing and purification of enzymes.

The enzymes used in the optical analyses should be highly active. This makes it possible to use only a few micrograms and yet obtain a rapid reaction which would be finished, within a relatively short interval of time. The initial rate of the reaction provides the best measure for the enzyme activity. In most cases concentrations ranging from 2 to 20 μg. protein per ml. were used. A few enzymes, such as the purified adenosine deaminase, were active in concentrations lower than 0·1 μg. per ml.

The possibility of selecting particular wave-lengths, one at which the density during the course of the enzyme reaction increases, the other at which it decreases, is a feature which adds greatly to the specificity of the methods. Enzymatic methods performed by manometric technique, for instance, hinge exclusively on the specificity of the enzyme preparations used, and the occurrence of unspecific reactions is therefore not readily discovered. Unspecific reactions which do not cause changes in the ultra-violet absorption may not interfere at all with the differential ultra-violet spectroscopy. In case they do give rise to density changes in the ultra-violet they are, however, readily discovered by comparing the density changes at various wave-lengths. It appears from Table 1 that there is a strict relationship between the density increase at one wave-length and the density decrease at another. Moreover, it is often possible to find wave-lengths at which no density changes take place during the reaction (isobestic points). A marked alteration between the theoretical ratio of density changes at selected wave-lengths and the found ratios at the same wave-lengths indicates that unspecific density changes are superimposed on the specific. This could be due to unspecific changes in the transparency during the enzymatic reaction or to the occurrence of other enzymatic reactions.

It should not be implied from what is said that mistakes cannot be encountered in enzymatic differential ultra-violet spectroscopy. This would, of course, not be a correct statement. It would not be an overstatement, however, to point out that the chances of getting into such complications as mentioned above have been greatly reduced by the use of differential ultra-violet spectroscopy at selected wave-lengths, using purified enzymes.

A further opportunity for a check of the specificity of enzymatic ultra-violet spectroscopy lies in the fact that the end-products found, in many cases, can be analysed once more by another specific enzyme. A concrete example will illustrate this. A solution containing 5·15 μg. adenosine per mol. was subjected to deamination in the presence of 1 μg. of purified adenosine deaminase. The total $\Delta \log (I_0/I)_{265}$ amounted to −0·156, which corresponds to complete deamination (1 μg. adenosine per ml. corresponds to a density decrease of −0·031). To the deamination product inosine (which has practically the same molecular weight as adenosine) is added nucleosidase, inorganic phosphate and xanthine oxidase, and the uric acid formed is measured by the density increase at 290 mμ. The total density increase found was +0·199, which corresponds to 2·62 μg. hypoxanthine per ml. being oxidized to uric acid, or as inosine 5·2 μg. Calculated: 5·15 μg.

One question more about the differential methods. It has been demonstrated that the methods are specific and that they give proportionality within a certain range of concentration. These density changes might be completely arbitrary, however, i.e. they need not have any relation to the differences in densities found by absolute ultra-violet spectroscopy of the compounds investigated or at least not be identical with the values obtained this way.

Table 2. *Density changes as measured by absolute and by differential enzymatic ultra-violet spectroscopy*

Reaction	Specific enzymes used	Density change by absolute or differential ultra-violet spectroscopy	$\Delta \log (I_0/I)$ for 1 μg. purine/ml.	
			290 mμ	265 mμ
Hypoxanthine to uric acid	Xanthine oxidase	Absolute	+0·080	—
		Differential	+0·076	—
Guanine to uric acid	Xanthine oxidase + guanase	Absolute	+0·048	—
		Differential	+0·047	—
Adenine to hypoxanthine (as ribosides)	Adenylic acid deaminase	Absolute	—	−0·056
		Differential	—	−0·052

If the $\Delta \log (I_0/I)$ obtained by absolute ultra-violet spectroscopy are compared with those found by the enzymatic differential ultra-violet spectroscopy, as it is done in Table 2, one will find that the differences are small and in some cases non-existent. In general, it can therefore be stated that the density changes found in enzymatic ultra-violet spectroscopy are practically identical with those found in absolute ultra-violet spectroscopy.

VI. APPLICATIONS OF THE MICRO-METHODS IN BIOLOGICAL MATERIAL

Proceeding now to the practical applications of the methods, the question arises whether the $\Delta \log (I_0/I)$ determined for a substance in a practically pure solution is also valid when studying biological material. The validity of the method as applied on biological material will appear from two examples. The first example illustrates a micro-uric acid determination in plasma (non-deproteinized) (Table 3), and the second a micro-adenylpyrophosphate analysis of a protein-free filtrate from a biopsy of rat muscle (Table 4). In both cases were added known amounts of the substance to be analysed, and in both cases the experimentally found amounts agreed closely with the added amounts. This means that the average constants

Table 3. *Micro-uric acid analysis of human plasma*

Plasma, 58 cu.mm. blown over in 2 ml. of three different solutions of 0·07 *M* glycine, *p*H 9·4, saturated with oxygen. Solution 1 contained no uric acid, solution 2 contained 1·85 μg. of uric acid in 2 ml., solution 3 contained 3·70 μg. of uric acid in 2 ml. By addition of plasma and of purified uricase at zero time the total volume was increased to 2·14 ml. Wave-lengths: 290 mμ (and 320 mμ).

	(1) Plasma, no extra uric acid	(2) Plasma + 1·85 μg. uric acid	(3) Plasma + 3·70 μg. uric acid
$\Delta \log (I_0/I)_{290}$ (corr. for blank)	−0·126	−0·187	−0·252
μg. uric acid*	3·75	5·64	7·50
μg. extra added uric acid		1·89	3·86

* Average density change at 290 mμ per 1 μg. uric acid per ml.: −0·072. 2·14 ml. containing 58 cu.mm. plasma contains 3·75 μg. uric acid; 100 ml. plasma therefore contains 6·45 mg. uric acid.

Table 4. *Micro-adenylpyrophosphate analysis of a muscle biopsy*

Muscle filtrate (perchloric acid) neutralized and buffered with 0·1 *M* succinate, *p*H 6·08 with or without added adenosine triphosphate (ATP). The added amount of ATP corresponded to 2·80 μg. adenyl radical (A.R.) per ml. of mixture. Enzymatic analysis with muscle deaminase and potato adenylpyrophosphatase plus calcium ions. Wave-lengths: 265 and 240 mμ.

	Control (no enzyme)	Mixture	Mixture + purified A.R. 2·8 μg./ml.
$\Delta \log (I_0/I)_{265}$	0·00	−0·050	−0·105
μg. A.R./ml.*	0	2·60	5·46
μg. A.R./ml. added	—		2·86†

* Average density change at 265 mμ per 1 μg. A.R. per ml.: −0·019.

† The amount of total acid-soluble phosphorus found in muscle filtrate was 2·48 μg. P per ml. The ratio A.R./total acid-soluble P was therefore approximately 1, which is approximately the same found from determination of pyro-P and total P according to Lohmann's method.

($\Delta \log (I_0/I)$) determined in pure solutions can be applied also for analysis of these substances in plasma or in filtrates from biological material.

Analysis of guanine in purine mixture from nucleic acid has also been performed; however, this analysis has not yet been subjected to the same kind of test as that described above. Since the analysis presumably is correct and a few questions in connexion with it might be of interest to discuss at this symposium, an example of such a type of analysis is presented.

VII. ANALYSIS OF GUANINE IN NUCLEIC ACID

Muscle nucleic acid was acid hydrolysed, the purines precipitated with copper bisulphite (Krüger's procedure), decomposed with H_2S and analysed as free purines.

A small aliquot (containing a few micrograms of guanine) was analysed at 290 mμ using +0·048 as the density change per μg. guanine per ml., with *guanase* and *xanthine oxidase* as analytical tools.

Total amount of guanine found: 3·82 mg.

A large aliquot was determined for Kjeldahl N. Found: 3·7 mg. N.

If all the N found were considered a mixture of equimolar amounts of adenine and guanine N the calculated guanine N would be 2·06 mg., therefore the total amount of guanine calculated from N is 4·40 mg.

The discrepancy between the 3·82 mg. guanine found directly and the 4·40 mg. guanine calculated from total N is presumably to be attributed to the fact that the latter value which is calculated from the determined Kjeldahl N includes appreciable amounts of pyrimidine N, which has been partially liberated during hydrolysis.

A chemical deamination of adenine and guanine in nucleic acid with subsequent acid hydrolysis would make it possible to analyse the two purine components of ribonucleic acid in one step. This would constitute a highly sensitive chemical method for analysing nucleic acid. In this way less than 0·02 μg. nucleic acid P could be analysed in a 0·1 ml. volume.

VIII. THE BIOLOGICAL SYNTHESIS OF PURINE COMPOUNDS

The micro-methods outlined have been applied for various purposes, partly for purine analyses of micro-samples of plasma and tissue fluids and of biopsies of normal or pathological tissues, partly for the study of biological syntheses of purine compounds. Only the latter aspect will be discussed in the present communication. It is clear that it would be comparatively simple to conduct studies of the possible biological precursors of the purines in the tissues using the outlined technique; such a study has not as yet been performed but is planned.

The formation of 6-amino-purines from 6-hydroxy-purines is also an interesting problem. This has been studied with the ultra-violet micro-technique as well as with isotope technique. The first-mentioned approach has not yielded any positive information. It was, for instance, not possible to detect any formation of adenosine by adding amides, like glutamine or asparagine, to inosine incubated with kidney extract under anaerobic conditions. Arginine proved also inactive in this system.

Studies with isotopic nitrogen (N^{15}) conducted in collaboration with Dr Rittenberg (Kalckar & Rittenberg, unpublished) showed, however, that the 6-amino group of adenosine triphosphate from the muscles is rejuvenated with great rapidity. Seven to eight hours after administration of isotopic ammonium citrate to rats, the adenosine-triphosphate, analysed as crystalline adenylic acid, gave the following analyses. The 6-amino-N (obtained by means of the specific adenylic acid deaminase) contained 0·093 % excess N^{15}. The ring N (hypoxanthine) only 0·007 % excess N^{15} and the 6-amino plus ring N, i.e. the adenine, contained 0·021 % excess N^{15}, which is almost the same as the amount calculated from the two other values. In other words, the ring N is not rejuvenated during the period of 8 hr., whereas the 6-amino-N was rejuvenated to a very high extent. The glutamic acid of the protein had only attained an N^{15} concentration of 0·020 % excess N^{15} during the same period of time. The amide-N of the protein had an isotope concentration as high as 0·100 % excess N^{15} and could therefore be considered one of the possible ammonia donors of the inosinic acid in muscle. This is no proof, however; one can only say that the high N^{15} concentration puts the spotlight on the amide-N as the possible donor. Interestingly enough the purine of the nucleic acid from muscle turned out to have a comparatively high isotope concentration, 0·030 % excess N^{15}.

IX. THE ENZYMATIC SYNTHESIS OF THE PURINE RIBOSIDIC LINKAGE

The glycosidic bond between ribose (or desoxyribose) and an imidazole nitrogen is a linkage characteristic of a number of biological substances, particularly the nucleosides, nucleotides and the nucleic acids. The enzymes which catalyse the fission of purine or pyrimidine nucleosides into free purines or pyrimidines and ribose (the so-called nucleosidases) have been known for a number of years. The nucleosidases have hitherto been classified as hydrolytic enzymes. If the purine (or pyrimidine) liberated is removed instantaneously, for instance, by enzymatic oxidation and if the medium contains reasonable amounts of inorganic phosphate the fission of the nucleoside proceeds to completion.

In the Institute in New York we recently found that purine nucleosidase is a phosphorolytic enzyme. In the absence of phosphate its activity dwindles to nothing and by addition of inorganic phosphate its activity is restored (see Fig. 4). This effect was actually described about 10 years ago by Klein (1935) who also found that arsenate acts likewise. The phenomenon was not understood at that time and it is only thanks to the fundamental work of Cori (Cori, Colowick & Cori, 1937) and his group that it is relatively simple to interpret such a phenomenon and therefore with comparative ease plan how to pursue it.

It was of primary importance to find out whether the liberation of purine was accompanied by an uptake of inorganic phosphate. The first results were definitely negative. However, the belief was entertained that these apparently negative results might be due to the fact that a new ester was formed which possessed an extremely high lability in acid solution and thus was completely hydrolysed during the procedure by which the phosphate colour is usually developed.

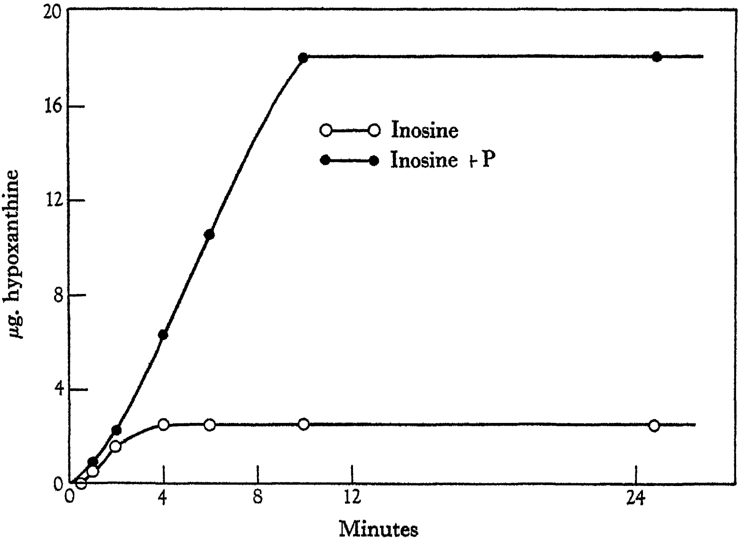

Fig. 4. The action of purified liver nucleosidase (in the absence of xanthine oxidase) on hypoxanthine riboside (inosine) in the absence and in the presence of 25 μg. of inorganic phosphate. Abscissae: incubation time in minutes. Ordinates: μg. hypoxanthine liberated (as determined in the filtrate by xanthine oxidase at 290 mμ).

By using a modified phosphate method recently introduced by Lowry (1946), a clear-cut proportionality between purine liberation and phosphate uptake was unravelled. In Lowry's method the phosphate colour is developed at *p*H 4 in acetate buffer using ascorbic acid as a reducer. This method is particularly useful for analysis of true inorganic phosphate in the presence of highly acid-labile esters such as amidinephosphates or acylphosphates or the new ester which is formed by enzymatic splitting of purine ribosides in the presence of phosphate.

By means of this method it was found that for each mol. hypoxanthine liberated from inosine 1 mol. of phosphate was taken up in organic linkage. It can be seen from Table 5 that phosphate was taken up only in the presence of a ribosidic bond and not merely by oxidizing hypoxanthine in the presence of free ribose. It was rather obvious, therefore, that the ribosidic linkage was split by inorganic phosphate, and that the latter was esterified either to the nitrogenous base or to the ribose component. The same was observed, using guanosine (guanine riboside) as the substrate. In this case 0·175 μmol. of purine was liberated and 0·168 μmol. P taken up.

Table 5. *Enzymatic phosphorolysis of inosine (hypoxanthine riboside)*

Substrates: 0·46 μmol. inosine plus 0·80 μmol. phosphate (control, 0·66 μmol. hypoxanthine plus 0·67 μmol. ribose). Enzymes: liver nucleosidase (20 μg.) plus xanthine oxidase (30 μg.). Buffer: glycyl-glycine, 0·05 *M*, *p*H 7·2. Gas: oxygen. Temp. 27°. Incubation time: 80 min.

	Uric acid formed μmol.	Phosphorus utilized μmol.
Inosine	0·25	0·27
Hypoxanthine + ribose	0·27	0·02

It has been possible to obtain appreciable amounts of the new ester by repeating on a larger scale the phosphorolytic experiment just described. The ester thus isolated proved to be extremely labile towards dilute strong acids. About 20–25% of the ester was hydrolysed after 5 min. at 30° in 0·2*N* hydrochloric acid. The ester contains 1 mol. pentose per mol. acid-labile P and is non-reducing. After a 15–20 min. hydrolysis in dilute hydrochloric acid at 30° the reduction power, measured as aldose, increased markedly. The ester, upon mild acid hydrolysis, liberated 0·8–1 aldose equivalent per mol. labile phosphate hydrolysed.

The ester isolated was considered as being a ribose-1-phosphate for the following reasons: (1) phosphate is involved in the splitting of the nitrogen aldehyde linkage of a purine riboside; (2) one aldehyde group is liberated per mol. acid-labile phosphate hydrolysed; (3) the high sensitivity of the ester towards acids and the somewhat higher stability in alkali; (4) the successful enzymatic synthesis of purine ribosides from the new ester plus purine, a reaction which will be described in the next section. It has yet to be demonstrated whether the sugar component is *d*-ribose, and whether the ester is an α or a β ester. It has recently been shown that the natural adenine nucleosides belong to the β-series (Todd, 1946).

The phosphoribosidic linkage, although highly acid-labile, is not affected as much by the presence of molybdate as is, for instance, phosphocreatine or acetylphosphate. It appears from Table 6, however, that molybdate does increase the rate of acid hydrolysis of ribose-1-phosphate to some extent.

If ribose-1-phosphate was incubated with equimolar amounts of hypoxanthine or guanine and liver nucleosidase, the free purine disappeared and was accounted for as purine riboside and the labile phosphate was liberated, 1 mol. P per mol. purine being taken up in ribosidic linkage (cf. Table 7). The equilibrium is very favourable towards the formation of hypoxanthine riboside. From Fig. 5 it can be seen that in the samples in which all the components were incubated only about 20% of the free hypoxanthine added (as determined by xanthine oxidase at 290 mμ) remained. The rest, 80%, was accounted for as hypoxanthine riboside (as determined by xanthine oxidase, nucleosidase and varying amounts of inorganic phosphate).

Table 6. *The effect of molybdate on the hydrolysis of ribose-1-phosphate in strongly acid solution*

Incubation time: 2 min. (samples 1 and 2) and 6 min. (samples 3 and 4). Temp. 30°.

Type of hydrolysis	μg. P liberated	% hydrolysed
(1) With molybdate in 0·1 N H_2SO_4	0·42	14·0
(2) With 0·1 N H_2SO_4	0·29	9·7
(3) With molybdate in 0·1 N H_2SO_4	0·99	33·0
(4) With 0·1 N H_2SO_4	0·76	25·3

Table 7. *Enzymatic synthesis of inosine (hypoxanthine riboside)*

Hypoxanthine, 0·58 μmol. plus ribose-1-phosphate, 0·58 μmol. are incubated with liver nucleosidase. In the controls either one of the three components was omitted during the incubation and added after deproteinization.

	Min. incubation	μmol. P liberated	μmol. inosine formed	μmol. hypoxanthine remaining
Control	40	0	0	0·53
Complete	20	0·32	0·39	0·10
Complete	40	0·36	0·38	0·11
Control	40	0	0	0·53
Control	40	0	0	0·51

Table 8. *Rate of synthesis of inosine*

38 μg. hypoxanthine (approx. 0·30 μmol.) incubated with ribose-1-phosphate (R-1-P) containing 9·35 μg. labile P (0·30 μmol.) and nucleoside phosphorylase (1 mg. protein). In the controls hypoxanthine or R-1-P added after deproteinization. Temp. 30°. pH 7·5.

	Min. incubated	μg. hypoxanthine	
		Free	Ribosidic bound
(1) R-1-P	15	35·2	0
(2) R-1-P + hypoxanthine	5	25·6	10·4
(3) R-1-P + hypoxanthine	30	7·2	24·0
(4) Hypoxanthine	30	33·6	0

The equilibrium at pH 7·5, with equimolar amounts of hypoxanthine and ribose-1-phosphate, and in the complete absence of inorganic phosphate, approximated 90% as hypoxanthine riboside and free phosphate and 10% as phosphate riboside and free hypoxanthine. The equilibrium reaction can be formulated in analogy to Cori's equation for polysaccharide synthesis (Cori, Cori & Green, 1943) and Doudoroff's equation for disaccharide synthesis (Doudoroff, 1943) as follows:

ribose-1-hypoxanthine + phosphate $\rightleftharpoons$ ribose-1-phosphate + hypoxanthine.

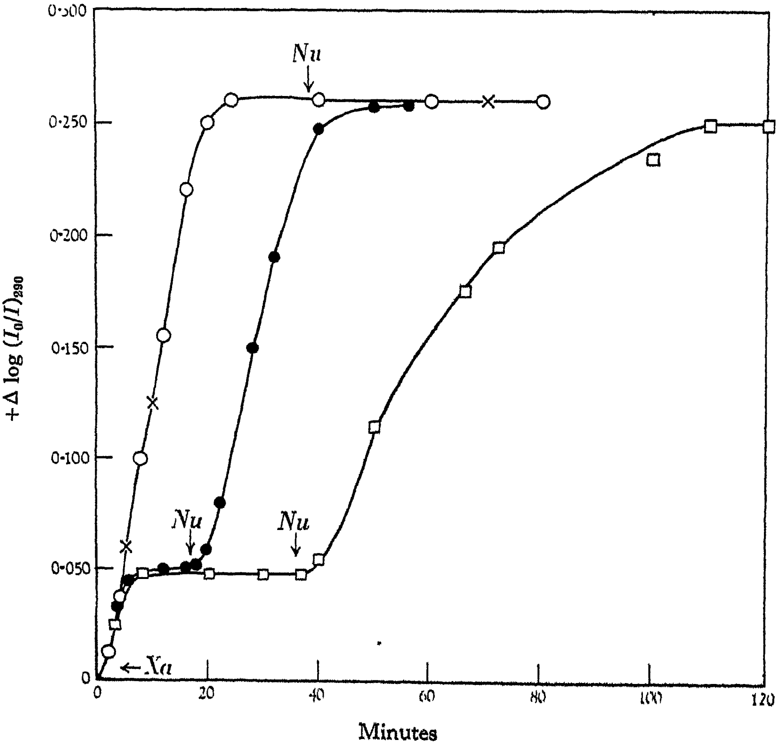

Fig. 5. Analysis for free and ribosidic bound hypoxanthine in filtrates from mixtures of equimolecular amounts of ribose-1-phosphate and hypoxanthine, incubated with nucleoside phosphorylase. In the controls the component omitted was added after deproteinization. ○, control without hypoxanthine; ×, control with ribose phosphate hydrolysis products instead of ribose-1-phosphate; □, complete sample, with 50γ inorganic phosphate added for inosine analysis; ●, the same with 300γ inorganic phosphate. *Xa*, xanthine oxidase added to react with free hypoxanthine; *Nu*, nucleoside phosphorylase and inorganic phosphate added for inosine determination.

The enzyme catalysing this equilibrium is a true phosphorylase, and it has therefore been suggested (Kalckar, 1945) to change the name from 'nucleosidase' to the more appropriate 'nucleoside phosphorylase'. While the extent of the hypoxanthine riboside synthesis is usually short of 90% (in most cases it proceeds to 70 or 80%), it is most likely due to the fact that ribose-1-phosphate is slowly being decomposed and in this way liberates small amounts of inorganic phosphate which are difficult to remove.

The synthesis of hypoxanthine riboside as catalysed by purified liver nucleoside phosphorylase proceeds with an appreciable velocity. In Table 8 it can be seen that already after 5 min. almost one-third of the hypoxanthine added (or 40% of the total synthesis) has been converted into the riboside in the presence of 1 mg. of the enzyme.

It has also been possible to demonstrate a synthesis of guanosine (guanine riboside) from guanine and ribose-1-phosphate (Kalckar, 1947*d*). About 15–30% of the guanine added was found in ribosidic linkage. It has only been possible to demonstrate a formation of guanine riboside but not as yet to account in an accurate way for the free guanine due to the low solubility of this base.

In one experiment in which labile phosphate (ribose-1-phosphate) was determined as well as guanine riboside the following results were obtained. 1 μmol. of guanine was incubated with 0·5 μmol. of ribose-1-phosphate (containing some inorganic phosphate) and nucleoside phosphorylase (free of guanase) for a period of 30 min. at 30°. Only 0·3 μmol. of free guanine was accounted for in the controls. In the complete sample 0·38 μmol. of ribose-1-phosphate disappeared, i.e. 76%, whereas only 0·27 μmol. of guanine riboside was accounted for. In the controls with ribose-1-phosphate or guanine and enzyme no ribose-1-phosphate disappeared. However, inorganic phosphate disappeared in the complete sample as well as in the sample in which guanine and enzyme were incubated together. The fate of the inorganic phosphate in these two samples is unknown.

It is, however, clear that the phosphorolytic reaction found for hypoxanthine compounds applies also for guanine compounds.

X. THE BIOLOGICAL SIGNIFICANCE OF NUCLEOSIDE PHOSPHORYLASES

The ability to synthesize purine ribosides, the so-called nucleosides, is a process which is widespread in mammalian tissues.

The spleen contains a desoxyribosidase (Klein, 1935) which might also turn out to be a synthesizing enzyme. The existence of ribosidases and desoxyribosidases of pyrimidine nucleosides in bone-marrow (Deutsch & Laser, 1929) may also be of interest in this connexion.

The requirement of *Hemophilus parainfluenza* for pyridine nucleotides (Lwoff & Lwoff, 1937) or nicotinamide ribosides (Gingrich & Schlenck, 1944) as growth factors and the inability to use free nicotinamide might be due to a lack of a particular nucleoside phosphorylase or inability to form ribose-1-phosphate (cf. Lipmann, 1943). The latter is less likely, since the organism apparently does not require purine nucleosides. The nature of the coenzyme-destroying enzyme which presumably splits off nicotinamide from reduced cozymase (Handler & Klein, 1942) need also further elucidation, particularly with respect to the problem of whether this process is a hydrolysis or a phosphorolysis.

Of particular interest are the recent observations of Colowick & Price (1946), in which they described a phosphorolytic enzyme which acts on ribonucleic acid and liberates guanine. This reaction which seems to be reversible has been formulated as follows:

Ribose-1-guanine nucleic acid (ordinary nucleic acid) + phosphate
$\rightleftharpoons$ ribose-1-phosphate nucleic acid (de-guanine nucleic acid) + guanine.

Colowick & Price have the following evidence for this reaction. If an enzyme prepared from muscle is added to ribonucleic acid (purified yeast nucleic acid) in the presence of inorganic phosphate, guanine is gradually released and the acid-insoluble residue contains less guanine and an equivalent amount of highly acid-labile phosphate. If inorganic phosphate is removed the guanine decreases in the acid filtrate and increases in the acid-insoluble residue.

It would be of great interest to study this reaction further and to investigate the possibility of the existence of an analogous reaction with desoxyribonucleic acid.

The observation that nucleic acid can undergo a phosphorolytic cycle by which phosphate is exchanged for a nitrogenous base may be of importance in understanding the biological action of purine compounds and nucleic acids. It is only fitting at Cambridge to recall the recent observations of Sir F. G. Hopkins (Hopkins & Simon-Reuss, 1944–5), that certain purines added to tissue cultures greatly increase the number of mitoses of connective tissue.

It is also of interest in this connexion to mention that Colowick & Price (1945) found that guanine is an activator of the muscle hexokinase (i.e. the enzyme which catalyses the phosphorylation of hexoses by adenylpyrophosphate). The liberation of guanine by phosphorolysis of nucleic acid therefore initiates the carbohydrate metabolism, at least in muscle tissue. This observation is worth bearing in mind in connexion with biological phenomena involving activity of the nuclear substances, as in the fertilization of the egg which is accompanied by a sudden burst in carbohydrate metabolism (Warburg, 1915), and the greatly increased aerobic and anaerobic carbohydrate metabolism observed in rapidly growing tissues (Warburg, Posener & Negelein, 1924; Lipmann, 1933).

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THE MACROMOLECULAR BEHAVIOUR OF NUCLEIC ACIDS

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I. THE ISOLATION OF NUCLEIC ACIDS

The preparation of a nucleic acid in an undegraded state necessarily precedes the investigation of its macromolecular behaviour. Gulland, Barker & Jordan (1945) have pointed out that the direct isolation of nucleic acids from tissues may lead to a degraded product, and they suggest that a more satisfactory procedure is to isolate and subsequently decompose the nucleoprotein. The desoxypentose nucleic acid of the thymus gland has been the subject of many investigations into the macromolecular structure and behaviour of nucleic acids (Hammarsten, 1924; Signer, Caspersson & Hammarsten, 1938; Astbury & Bell, 1938; Stenhagen & Teorell, 1939; Greenstein & Jenrette, 1940, 1941; Pedersen, 1940; Cohen, 1942; Greenstein, 1943; Tennent & Vilbrandt, 1943; Vilbrandt & Tennent, 1943), and an extensive study has been made of the ribonucleic acid of the tobacco mosaic virus by Cohen & Stanley (1942).

The method of isolation of the desoxypentose nucleic acid of the thymus gland generally employed in the above investigations is that originally suggested by Bang (1903) and later developed by Hammarsten (1924). The analysis of the acid isolated by Hammarsten, however, shows that the nitrogen and phosphorus content (N, 11·97; P, 7·09%) was low when compared with the theoretical analysis for the tetrasodium salt of a desoxypentose polynucleotide consisting of the four nucleotides in equivalent proportions (N, 15·85; P, 9·37%). Furthermore, later preparations obtained by this method were found to contain a small amount of protein (Signer *et al.* 1938; Gulland, Jordan & Taylor, 1946), and it is possible that the Hammarsten-Bang procedure may not be a satisfactory method of isolating nucleic acids. An alternative procedure, therefore, has been developed (Gulland, Jordan & Threlfall, 1946) which leads to a more satisfactory product, as is shown by the studies described in this paper. The method makes use of the remarkable differences in solubility of nucleoproteins in sodium chloride solutions of different strengths which have been described by Mirsky & Pollister (1942, 1943) and of the procedure for the removal of protein introduced by Sevag, Lackman & Smolens (1938). It is an important feature of this preparation that the solutions at no time deviate significantly from *p*H 7·0. The product obtained, unlike that prepared by the Hammarsten-Bang method, was free from protein as shown by the absence of a gel on shaking with a chloroform-amyl alcohol mixture and by a negative Sakaguchi reaction.

II. THE CHANGES PRODUCED IN THE SODIUM SALT OF DESOXYPENTOSE NUCLEIC ACID BY TREATMENT WITH ACID AND ALKALI

The electrometric titration of the sodium salt of the desoxypentose nucleic acid of the calf thymus gland, isolated as described above, has been carried out by Gulland, Jordan & Taylor (1946) (Fig. 1). When acid or alkali is added to the solution in water no groups are titrated at first between *p*H 5·0 and 11·0, but outside these limits a rapid liberation of groups titrating in the range *p*H 2·0–6·0 and *p*H 9·0–12·0 respectively takes place. On back-titrating either with acid from *p*H 12·0 or with alkali from *p*H 2·5, a different curve is obtained from that produced by the initial titration, and it is significant that the same curve is obtained whether the back-titration is with alkali from *p*H 2·5 or with acid from *p*H 12·0. The nature of the acidic and basic groups of the desoxypentose nucleic acid of calf thymus has been discussed by Gulland, Jordan & Taylor (1946), and it is concluded that the groups titrated between *p*H 8·0 and 12·0 are the enolic hydroxyl groups of guanine and thymine, and that those titrating between 2·5 and 6·3 are the amino groups of guanine, adenine and cytosine. Furthermore, four primary

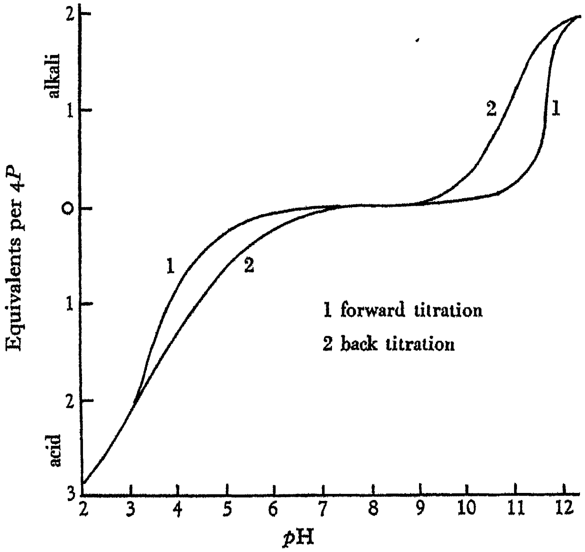

Fig. 1. Dissociation curve of desoxypentose nucleic acid of calf thymus.

phosphoric acid dissociations are present, as shown by sodium analysis, and thus the back-titration curve coincides almost exactly with the theoretical curve for a polynucleotide containing the four nucleotides in unimolecular proportions. The complete identity of the back-titration curves suggests that acid and alkali have the same effect in liberating amino and hydroxyl groups, and the simplest explanation of the effect described above would appear to be that in the original sodium salt of desoxypentose nucleic acid a linkage exists between the hydroxyl and amino groups. There are two possible ways in which this linkage may occur. First, it may be by hydrogen bonding, involving resonance between the two forms —$NH_3^+O^-$— and —NH_2HO— and possibly including water molecules as part of the bond. For steric reasons such bonding cannot occur between the amino and hydroxyl groups of the same base but must exist between nucleotides of the same or adjacent covalent chains. Hunter (1945) has pointed out that hydrogen bonding between acidic and basic groups will tend to break down when these groups are neutralized, which is in conformity with the observed liberation of groups on treatment with acid and alkali. Secondly, the linkage may be formed by a radical hitherto unidentified in the breakdown products of the nucleic acid molecule, which links the amino and hydroxyl groups. It should be mentioned, however, that such a radical could not contain groups which are titrated within the range *p*H 2·0–13·0, since the back-titration curve shows no liberation of such groups.

Solutions of the sodium salt of desoxypentose nucleic acid in water are extremely viscous, and it has been shown previously (Hammarsten, 1924; Vilbrandt & Tennent, 1943) that the viscosity decreases when the solution is made acid or alkaline. The viscosity of solutions of the sodium salt of desoxypentose nucleic acid prepared by Gulland, Jordan & Threlfall (1946) has been shown by Creeth, Gulland & Jordan (1946) to be much greater than that recorded previously (Greenstein & Jenrette, 1940, 1941; Vilbrandt & Tennent, 1943) for the acid isolated by the Hammarsten-Bang method; comparative values for relative viscosities at *p*H 7·0 are 5·7 (Vilbrandt & Tennent, 1943, 0·3 % solution at 25° C. measured in an Ostwald viscometer) and 116 (Creeth *et al.* 1946, 0·243 % solution at 25° C. measured in a Frampton (1939) viscometer at a pressure of 8000 dynes/cm.^{2}). Creeth *et al.* (1946) have also shown that in the variation of viscosity with change of the *p*H of the solution there are two highly critical *p*H values. The initial high viscosity of the solution at *p*H 7·0 is maintained as the *p*H is varied from 5·6 to 10·9, but in solutions more acid than *p*H 5·6 or more alkaline than *p*H 10·9 the viscosity falls sharply to a very low value. It is considered to be significant that these limits of *p*H are coincident with those observed for the liberation of acidic and basic groups (Fig. 2).

The solution at *p*H 7·0 exhibits marked streaming birefringence, and this is also diminished as the *p*H is changed and is absent in strongly alkaline or acid solutions (Fig. 3).

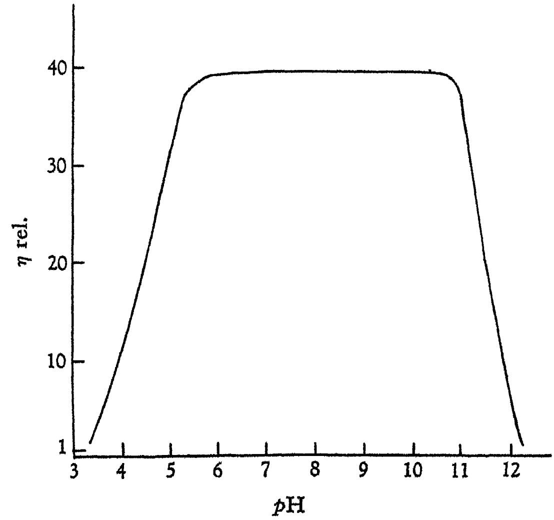

Fig. 2. Viscosity of thymus desoxypentose nucleic acid solutions (pressure $7 \cdot 10^3$ dynes/cm.^{2}).

The decrease in viscosity brought about by the addition of acid or alkali is considered by Vilbrandt & Tennent (1943) to be caused by a depolymerization of the desoxypentose nucleic acid. In our opinion such a depolymerization could not involve the rupture of the internucleotide ester linkages, since no increase in the amount of secondary phosphoric acid dissociation is observed in the back-titration curve, nor for the same reason could it involve the breaking of a P-N bond. Any depolymerization which occurs cannot therefore involve the rupture of a covalent bond. Nevertheless, the fall in viscosity is closely associated with the liberation of the amino and hydroxyl groups which are blocked in the original nucleic acid, and the titration evidence, as has been pointed out above, strongly suggests that the blocking of these groups is caused by a form of linkage between them. This liberation of hydroxyl and amino groups could either be brought about by a rearrangement within the molecule or by a depolymerization of the nucleic acid. The former process would, however, almost certainly lead to a more asymmetric structure, a result which is not compatible with the observed loss of streaming birefringence in acid and alkaline solutions. It would

*p*H	3·7	4–4·3	4·3–5	5–10	10–10·7	10·9–11·6	12
S.B.	0	1	2	3	2	1	0

Fig. 3. Streaming birefringence of a 0·24 % solution of desoxypentose nucleic acid (ionic strength 0·01).

appear, therefore, that the latter explanation in which the micelle is considered to disaggregate into smaller molecular units by rupture of the linkage between the amino and hydroxyl groups is more in conformity with the titration, viscosity and streaming birefringence data. This hypothesis may be compared with that evolved by Cohen & Stanley (1942) to explain the behaviour of the ribonucleic acid of tobacco mosaic virus. If this suggestion is confirmed by ultra-centrifuge studies now in progress it would appear that the molecular species in solution at *p*H 3·0 and 12·5 represent what is generally referred to as a desoxypentose nucleic acid molecule consisting of nucleotides covalently linked into polynucleotides, not necessarily all of the same size and whose mean molecular weight has yet to be determined, and containing in the aggregate four primary phosphoric acid dissociations, three amino dissociations and two enolic hydroxyl dissociations for every four atoms of phosphorus.

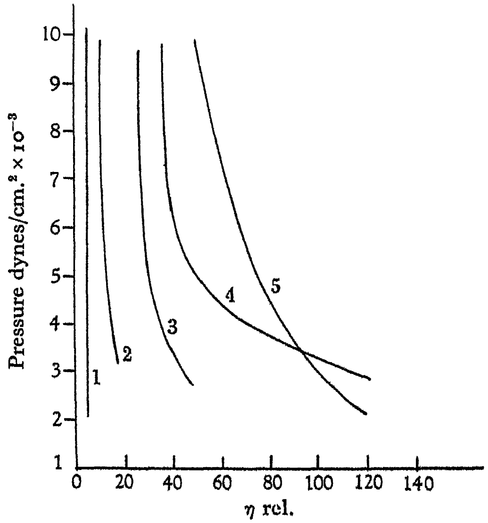

Fig. 4. Increase of the viscosity of solutions of sodium salt of desoxypentose nucleic acid after alkaline treatment. (1) 25 min., (2) 18 hr., (3) 42 hr., (4) 91 hr., (5) original.

If a solution of the sodium salt of thymus desoxypentose nucleic acid is allowed to stand at *p*H 7·0 after treatment with alkali at *p*H 12·5, there is a gradual increase in the viscosity (Vilbrandt & Tennent, 1943; Creeth *et al.* 1946) which after 96 hr. approaches that of the original solution of desoxypentose nucleic acid at *p*H 7·0 (Fig. 4). The viscosity of this solution, however, has a greater structural character than that of the original solution, being less than the original at high pressures and greater at low pressures. The behaviour on treating with acid at *p*H 3·0 and standing at *p*H 7·0 is somewhat different, a 0·243 % solution showing only a small increase in viscosity after

96 hr., whereas a 1·0 % solution gelled after 12 hr. Electrometric titration of the solution of the sodium salt of desoxypentose nucleic acid when left at *p*H 7·0 for 96 hr. after alkaline or acid treatment gives an identical titration curve to that obtained on back-titrating immediately after the alkaline or acid treatment, thus indicating that the increase of viscosity, which is most probably a repolymerization, does not involve the blocking of the amino and enolic hydroxyl groups in the same manner as in the original acid. This observation is not altogether incompatible with the possibility that the repolymerization does involve a bonding between the amino and hydroxyl groups as is postulated for the original acid, since the micelle produced would almost certainly contain water molecules as an integral part of its structure, permitting the approach of hydrogen and hydroxyl ions to the groups involved.

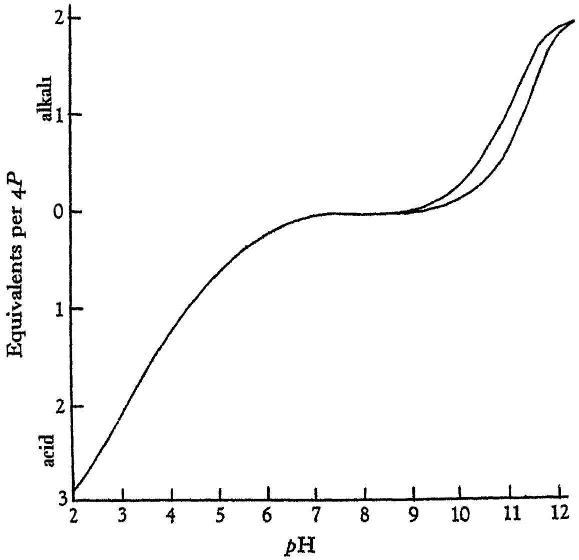

Fig. 5. Dissociation curve of alkali treated desoxypentose nucleic acid.

Precipitation of the sodium salt of desoxypentose nucleic acid by the addition of ethyl alcohol at *p*H 7·0 after acid or alkaline treatment gives a product which exhibits some blocking of the hydroxyl groups but not of the amino groups (Fig. 5). This observation cannot at present be explained, although if the above picture of the repolymerization is correct the dehydration of the micelle by alcohol treatment will almost certainly bring the reacting groups into closer contact, and thus form a stronger bond. It would appear, however, that the structure of the precipitated material is more dependent on the process of precipitation than upon the age of the solution, as the titration curves of samples precipitated immediately and after 96 hr. gave almost identical titration data.

III. THE CHANGES PRODUCED IN SOLUTIONS OF THE SODIUM SALT OF DESOXYPENTOSE NUCLEIC ACID BY THE ADDITION OF NEUTRAL SALTS

The viscosity of a solution of the sodium salt of desoxypentose nucleic acid is reduced by the addition of neutral salts. The reduction is considerable at low salt concentrations, and a critical concentration (approximately 0·01 *M* in the case of sodium chloride) has been observed by Creeth *et al.* (1946), such that any increase of the concentration above the critical produces relatively only a small change in viscosity (Fig. 6). A slight maximum is observed in the viscosity at approximately 1 *M*, a result which is similar to that observed by Needham, Kleinzeller, Miall, Dainty, Needham & Lawrence (1942) for the action of salts on the viscosity of solutions of myosin. The observations of Greenstein & Jenrette (1940, 1941) were conducted at salt concentrations greater than the critical. Creeth *et al.* (1946) found only a slight decrease in the streaming birefringence on the addition of sodium chloride to give a 4*M* solution, whereas Greenstein & Jenrette (1940, 1941) observed a disappearance of streaming birefringence on the addition of sodium chloride to this concentration with the sample of desoxypentose nucleic acid studied by them, and suggested a reversible depolymerization process as the explanation of the fall in viscosity on the addition of a neutral salt, the guanidine ion being particularly effective. By titrating the sodium salt of desoxypentose nucleic acid in the presence of 1 *M* potassium

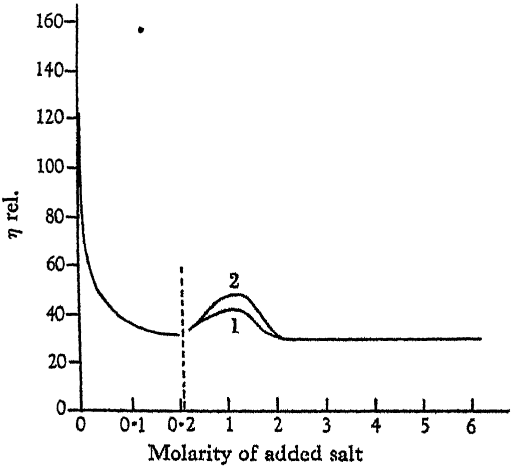

Fig. 6. The effect of sodium chloride (1) and guanidine sulphate (2) on the viscosity of the sodium salt of desoxypentose nucleic acid 0·24% (pressure $8 \cdot 10^3$ dynes/cm.^{2}).

chloride and in 5·06 M guanidine sulphate (molar with respect to the guanidine ion), Gulland, Jordan & Taylor (1946) showed, however, that even in the presence of these salts blocking of the hydroxyl groups persisted (Figs. 7, 8). The change in the viscosity on the addition of neutral salt thus bears no relationship to the depolymerization which occurs on the addition of acid or alkali. The change in viscosity could be attributed to one of at least three processes, viz. a disaggregation of coarse aggregates of micelles, a change in shape of the micelle, or changes in the ion atmosphere and hydrosphere. The last would appear to be the more plausible, but the data so far obtained on the electric mobility of the desoxypentose nucleate ion or micelle (Stenhagen & Teorell, 1939, Cohen, 1942) does not permit an assessment of this effect to be made at present.

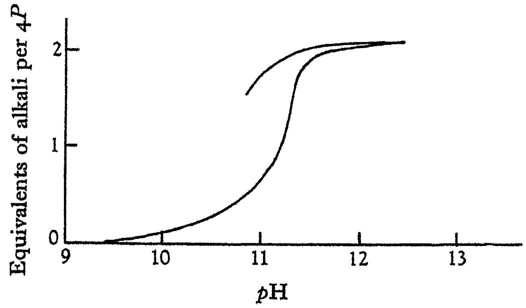

Fig. 7. Dissociation curve of desoxypentose nucleic acid in 1 m. KCl.

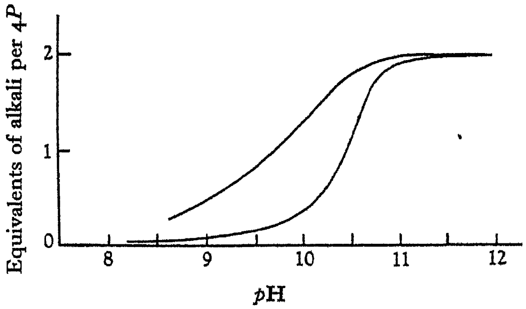

Fig. 8. Dissociation curve of desoxypentose nucleic acid in 5·06 m. guanidine sulphate.

IV. CONCLUSION

It is appreciated that the data so far collected have been obtained by studying a sample of desoxypentose nucleic acid which has passed through a drying process. Such a stage in the method of isolation has very probably produced some changes in the macromolecular structure that existed in the nucleoprotein in the cell. It is an obvious extension of this work to study a solution of desoxypentose nucleic acid which has been isolated without dehydration.

The change in structure of the desoxypentose nucleic acid of thymus gland in acid and alkaline solutions may be likened, if the picture of the macromolecular structure postulated above is correct, to the process of protein denaturation postulated by Wu (1931) and elaborated by Mirsky & Pauling (1936). Changes in the titration curves of protein on denaturation, which bear some resemblance to the data recorded above for desoxypentose nucleic acid in that there is a change in the number of groups titrated, have been recorded by several workers (see, for example, Chou & Wu, 1934; Theis & Jacoby, 1943).

At present there appears to be no direct explanation of the differences in behaviour and structure observed between the samples of desoxypentose nucleic acid isolated by the Hammarsten-Bang method and by the method outlined in this paper, although it is significant, considering the marked effect of *p*H in the macromolecular behaviour of desoxypentose nucleic acid, that the aqueous solution of the nucleic acid used by Vilbrandt & Tennent (1943) was at *p*H 5·60 (0·3 %), whereas that isolated by Gulland, Jordan & Threlfall (1946) gave a *p*H of 6·90 (0·243 %). An observation by Cohen (1945), however, suggests a more fundamental explanation. The viscosity of a solution of thymus nucleohistone increases as the protein is removed by the enzymes chymotrypsin and trypsin, and as an explanation it was suggested that the nucleic acid molecules aggregated when set free from the nucleoprotein. This raises the problem whether the mode of separation of the nucleic acid from the protein, e.g. by precipitation of the nucleic acid in bulk, by concentration of the protein at an interface, by an enzymic degradation of the protein or by electrophoresis, may have an influence on the particular form of aggregation which occurs. This may be revealed when an extensive investigation of the properties of thymus nucleoprotein, which has been commenced, are completed.

Further studies of the desoxypentose nucleic acid of the thymus gland employing ultra-centrifuge and electrophoresis methods are in progress.

We wish to acknowledge our indebtedness to Mr H. F. W. Taylor, B.Sc., and to Mr J. M. Creeth, B.Sc., with whom we have discussed many points in this paper.

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X-RAY STUDIES OF NUCLEIC ACIDS

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There is not a great deal that is recent in the experimental work reported in this paper, for there has been little opportunity to go on with the nucleic acids until the last few months, but our earlier publications (Astbury & Bell, 1938) included only part of our findings, and this is a very suitable occasion to look at them all again from a more up-to-date viewpoint in order to see how we stand before renewing the attack.

In the eyes of the structure analyst at least, perhaps the most significant physical fact about the nucleic acids is their high density. On drying *in vacuo* over P_2O_5 we have found the following values: Na thymonucleate, 1·63 g./c.c.; yeast nucleic acid and tobacco mosaic virus nucleic acid, 1·65 g./c.c. Now if we set out the chemical formulae for the various nucleotides and estimate their average area from known atomic dimensions, or better still, if we construct scale models, we see that this average area amounts to about $13\frac{1}{2}$ by 8 A., or something of the order of 100 A.^{2} If therefore the nucleotides lie on top of one another, or in some arrangement that in terms of packing comes to the same thing, we have:

$$T \times (13\tfrac{1}{2} \times 8) \times 1{\cdot}63 = 1{\cdot}65 \times 330, \quad \text{i.e.} \quad T \approx 3{\cdot}1\ \text{A.},$$

where T is the effective thickness and 330 is the average weight of a sodium nucleotide, and $1{\cdot}65 \times 10^{-24}$ g. is one-sixteenth of the mass of an oxygen atom. By this single calculation we are at once informed of the essentials of the problem; for, approximate as it is, it leaves no doubt that the distance of separation of successive nucleotides must be uncommonly small, and it reminds us of graphite, the *flat* networks of which are 3·4 A. apart, and even more of ascorbic acid (Cox, 1932; Cox & Goodwin, 1936), the *flat* rings of which are only 3·16 A. apart and whose density is as high as 1·74 g./c.c. The purine and pyrimidine components of the nucleotides must be flat, or nearly so, because of their double-bond structure, and so it follows with a high degree of probability that (*a*) the sugar component also must be flat, or nearly so, and parallel to the purine or pyrimidine, i.e. it must be a furanose, not a pyranose, sugar; and (*b*) that the linkages between base and sugar must be either all α or all β.

Another small piece of preliminary arithmetic brings in the flow properties of Na thymonucleate solutions investigated by Signer, Caspersson & Hammersten (1938), who concluded that the units are rods about 300 times as long as they are thick and of weight between 600,000 and 1,000,000. If the units are single columns of nucleotides their weight should be about $(300 \times 13\frac{1}{2})/T \times 330$, i.e. of the order of half a million. Probably some of the units in solution are aggregates of columns, so the agreement is satisfactory enough.

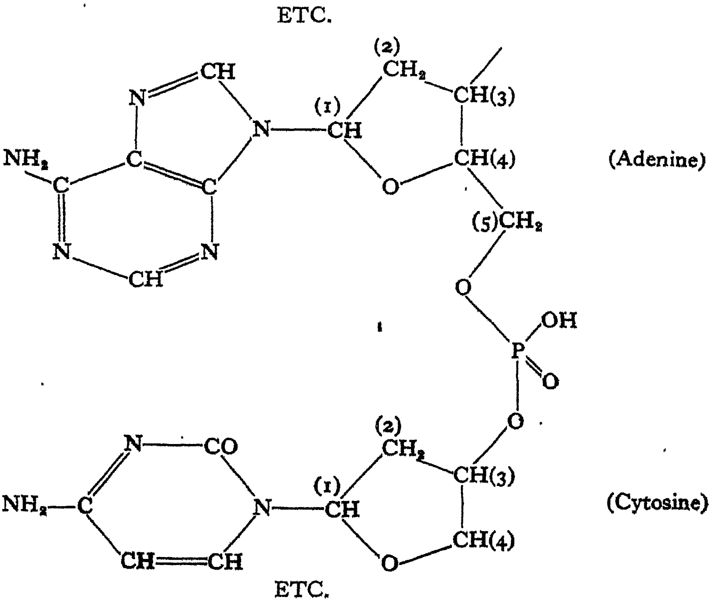
If we remember too that both fibres (Schmidt, 1937) and flowing solutions (Signer *et al.* 1938) of Na thymonucleate show strong birefringence that is negative with respect to the fibre axis or the direction of flow, then, before approaching any X-ray study, we already have a pretty firm general impression of what the answer is going to be. The molecules of Na thymonucleate and other polynucleotides of similar high density are presumably stiff columns of nucleotides fitting closely on top of one another.

X-ray studies more or less clinch this conclusion and give it quantitative expression. There is no need to describe again the experimental side of the Na thymonucleate investigation, but Pl. 1, fig. 1 is now presented as a better oriented fibre photograph than any we have published before. Unfortunately, the degree of orientation attained is still not high enough to allow the unit cell to be determined with certainty, and we prefer to postpone any definite statement in this respect pending the outcome of further experiments in progress. What is clear, however, is this, that the pattern repeats along the axis of the molecule at a distance corresponding to the thickness of eight nucleotides or a multiple of eight nucleotides—most probably eight or sixteen nucleotides. The least possible value of the fibre period is about 27 A., which would make the effective average thickness of a nucleotide about 3·4 A. The important point here has to do with the tetranucleotide constitution of the molecule, and in this connexion I wish to leave no doubt as to just what the X-ray diagram is so far telling us. It says simply that *the arrangement in space* is repeated along the fibre axis every eight (or sixteen) nucleotides. It says nothing yet, without very much more investigation, about the nature of the sequence, which may be determined by either chemical or geometrical considerations, or a combination of both. It hardly seems likely, though, that the fact that the intramolecular pattern is found to be based on a multiple of four nucleotides is unrelated to the conclusion that has been drawn from chemical data that the molecule is composed of four different kinds of nucleotides in equal proportions. It seems improbable, too, to judge by the degree of perfection of the X-ray fibre diagram, that these four different kinds of nucleotides are distributed simply at random; rather must they follow one another in some definite order—at least, in the more crystalline regions of the structure that give rise to the regular diffraction pattern. It is necessary to make this proviso, because in high polymeric aggregates there is always the possibility that there are long portions of chains (or columns in this case) of sufficient regularity to build up crystallographically more perfect regions, while at the same time there occurs every so often some chemical peculiarity that might indeed confer specificity on a structure that is otherwise designed to perform some more standard function. These things are matters for future research, and for the moment we must content ourselves with the statement that present X-ray evidence indicates that probably the bulk of a Na thymonucleate fibre is constructed from molecules built to a regular pattern, geometrical or chemical or both, based on a sequence of nucleotides that is a multiple of four.

As emphasized in previous communications (Astbury & Bell, 1938), the outstanding feature of the Na thymonucleate diffraction pattern is the strong meridian arc of spacing 3·34 A. that we have associated with a succession of not very dissimilar flat nucleotides lying transversely all along the molecular column. We have found no sufficient reason to modify this view, and taking everything into account it still seems by far the most satisfactory interpretation. It is not yet certain, however, till more highly oriented preparations have been attained, whether the arc lies truly on the meridian, whether it consists of two overlapping layer-line reflexions, or whether—as seems most likely—it combines both these manifestations. Crystallographically speaking, the point is of no great importance at the present stage of trying to deduce the main outlines of the structure: it only means that it is as yet uncertain whether the average thickness of a nucleotide is 3·34 A. or a little bit more, say 3·4 A.

It might be asked on what grounds has it been inferred that the nucleotides lie immediately on top of one another like a great pile of plates and are not disposed spiralwise round the long axis of the molecule. Arrangements of the latter kind are certainly not ruled out in general and in reactions with other molecules such as the proteins, but we are dealing here with the equilibrium arrangement, or state of crystallization, of solid Na thymonucleate, and the overriding consideration, it must be repeated, is the high density. If successive nucleotides do not lie closely on top of one another, then the state of packing must be dimensionally equivalent to such an arrangement, that is to say, nucleotides of neighbouring molecules must be *closely* interleaved in a surprisingly regular fashion. This seems highly unlikely, for the stabilizing forces of the structure will be chiefly the strong total attractions between the large flat areas, and the simplest and most effective way of reducing the potential energy to a minimum is for such areas to lie, if possible, directly on top of one another, as happens in other structures built from flat units. The stiffness and the estimated thickness (see Greenstein, 1944, p. 200, table) of rod-like units that have been inferred from the flow properties of Na thymonucleate solutions also point in the same direction.

A test that cannot long be dispensed with in any enquiry into the structure of a complex molecule is that of trying to build an accurate atomic model on the basis of known sizes and inter-bond angles. Chemical formulae are no more than a convenient shorthand, and it is always revealing, and often startling, to see what a molecule looks like in space. Dr M. M. Davies has kindly built for me a short length of the postulated column of nucleotides, and though detailed description of this model is preferably postponed until better data are forthcoming about the dimensions of the unit cell, a few of the more interesting features should be mentioned. It is a compact edifice in which, as expected, all the ring structures are practically flat and parallel, but the pyrimidine and purine rings are not co-planar with the sugar rings (assumed to be β-*d*-desoxyribose throughout; see also papers by Todd and Lythgoe in this Symposium), because the glucosidic linkage joins base and sugar at an angle. There is no difficulty in bringing the bare nucleosides as close together as 3·4 A., but when they are transformed to linked nucleotides by double phosphorylation, then there arises a situation that is perhaps a little unusual. The linkage must be between C (3) and C (5) because there are no other hydroxyl groups available, and it therefore involves the 4-atom chain, —C—O—P—O—, thus:



(N.B. This formula is not meant to convey any idea of the spatial arrangement in the model. For example, the nucleotides lie transverse to the axis of the column, i.e. perpendicular to the plane of the paper.)

This chain forms a zigzag bridge from the sugar end of one nucleotide to the next that is quite satisfying so long as we consider only the covalent distances between atoms ($r_C = 0{\cdot}77$ A., $r_O = 0{\cdot}67$ A., $r_P = 1{\cdot}0$ A.); it is when we attach the O and OH to the phosphorus atom that the apparent anomaly arises. The van der Waals separation of oxygen atoms is usually quoted as about 2·7 A., but if we adhere to a *uniform* separation of this order successive nucleotides can hardly be brought nearer than about 4 A. There is no convincing reason, though, why we should do so in face of the total van der Waals attractions between the large flat ring systems, and it is a fair conclusion that these attractions are strong enough to distort the oxygen van der Waals fields somewhat, after the manner of the compression of methyl groups that takes place when isobutylene polymerizes (see calculation made by Woods & Astbury in Astbury, 1945 *a*). It is possible that this supposed distortion of the oxygen fields has the effect of making the polynucleotide column more labile than would otherwise be the case.

Right from the beginning of this X-ray study of the nucleic acids we have drawn attention to the fact that the spacing (3·34 A.) of the nucleotides along the thymonucleic acid column is to all intents and purposes the same as the distance from one side chain to the next along an extended polypeptide, and even at the risk of being accused of dabbling in numerology I should like to say again that I believe this to be no mere coincidence. In a sense the evolution of biological molecules has been one long story of numerology, a sifting and sorting of shapes and sizes and a progressive selection of molecular patterns that has been going on throughout the ages. Biosynthesis is supremely a question of fitting molecules or parts of molecules one against another, and one of the great biological developments of our time is the realization that probably the most fundamental interaction of all is that between the proteins and the nucleic acids. I wish to stress again that not only are the basic components of the nucleotides flat or nearly so, but also the chosen sugar is in the flat furanose configuration, and it is this combination that leads to the correspondence between inter-nucleotide spacing and inter-side chain spacing. I suggest that the agreement is not an arithmetical accident but a stereochemical correlation of deep significance.

The spacing of the nucleotides along the Na thymonucleate column is independent of the water content of the preparation (which we found to be of the order of 20 % at room humidity). The X-ray photograph of a specimen dried in a vacuum desiccator shows the meridian arc undisturbed, but the diagram as a whole is poor and ill-defined compared with what is obtained at ordinary humidity. The side spacings are seen to be shorter, but it is not possible to be sure in which direction or directions the shrinkage takes place—except, of course, that it is a lateral shrinkage. We obtained a similar result when we tried the experiment of spinning very fine fibres into warm alcohol by means of a spinneret. Some of these fibres were as fine as spider's web and we hoped that they would give a perfectly oriented X-ray diagram; but owing to the strong dehydrating action of the warm alcohol the photograph turned out to be one of the poorest we had ever obtained. It is evident that water molecules play an essential part in the stability of the crystalline aggregates built up by Na thymonucleate at ordinary humidity, in which respect they resemble the crystallites of alginic acid (Astbury, 1945*b*).

Pl. 1, figs. 2*a* and 2*b* show two hitherto unpublished X-ray comparison photographs of oriented Na thymonucleate and an oriented preparation of the tough fibrous compound that, following advice from Dr Albert Fischer of the Carlsberg Foundation, we made by interaction between an aqueous Na thymonucleate solution and a hydrochloric acid solution of the protamine, clupein. If the idea is sound that this interaction can take place without steric hindrance by fitting column and chain directly alongside each other, we might expect a strong meridian arc at $3{\cdot}3_4$ A. to dominate the fibre diagram of clupein thymonucleate just as it does that of the sodium compound, though there should be increases in the side spacings. Figs. 2*a* and 2*b* show how these expectations appear to be justified: the principal intramolecular period along the fibre axis, the period that is common to nucleotides and side chains alike, remains unchanged.

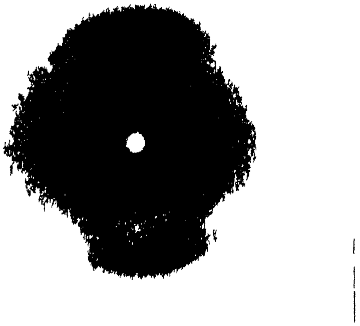
Dr K. Bailey kindly carried out for us some chemical analyses of this compound of clupein and thymonucleic acid and found the following: phosphorus, 5·65%; nitrogen, 21·8%; ash, 1·8%. These figures lead to about 57% thymonucleic acid, and 41% clupein, by weight. Now according to Linderstrøm-Lang (1935) and Rasmussen & Linderstrøm-Lang (1935) the clupein chain consists of some 20 arginine residues and 8 monoamino (or imino) residues, weighing in all about 4000, leading to an average residue weight of about 143; while according to Waldschmidt-Leitz and coworkers (Waldschmidt-Leitz & Kofranyi, 1935) it consists of 10 arginine residues and 5 monoamino (or imino) residues, weighing in all 2021, leading to an average residue weight of about 135. On the basis, therefore, of the Linderstrøm-Lang figures it follows that our clupein thymonucleate is composed of amino acid residues and nucleotides in the ratio (41 × 308) : (57 × 143), i.e. about 1·55 : 1; while on the basis of the Waldschmidt-Leitz figures the corresponding ratio is about 1·64 : 1. For strict chemical equivalence between the phosphoric acid groups of the nucleotides and the basic side chains of the protein we should expect 28 : 20, i.e. 1·4 : 1, from the Linderstrøm-Lang figures, and 15 : 10, i.e.

1·5 : 1, from the Waldschmidt-Leitz figures. In either case the agreement with Bailey's analyses is sufficiently close to permit the inference that for the most part the compound is formed in such a way that the phosphoric acid groups balance the basic side chains. Exactly how this is brought about it is hardly possible to say at the present stage of the investigation, but one conceivable mechanism would be for the long arginine side chains—they occur in sequences such as —*A*—*A*—*X*—*A*—*A*—*A*—*A*—*X*—*A*—*A*—*X*—, etc., where *A* stands for arginine and *X* for one or other of the monoamino (or imino) residues—all to curl round towards the row of phosphoric acid groups, while 'squeezing out' the inactive side chains in a succession of bulges in the other direction; or it may be that the polynucleotide column is actually depolymerized and that each arginine side chain then picks up a nucleotide by interaction between the basic 'fork' of the guanidine end-group and the *two* acid hydroxyl groups that are now free. It would be obviously well worth while to do a lot more X-ray and related chemical work in order to try to settle this question.

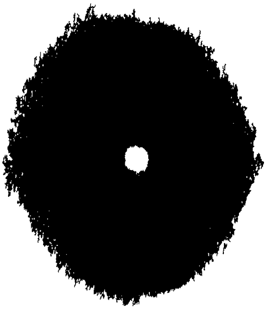
Turning now to nucleic acids of the ribose type it should first be noted that much the same high density prevails, and so much the same sort of close packing of the nucleotides is to be expected. Let it be said at once that in spite of many attempts we have not yet been rewarded with a fibre pattern by any specimen of this type so far available to us, and what follows is of an exploratory nature and rather along the lines of analogy. It describes, besides, results obtained mostly some years ago with preparations made by earlier techniques that are now suspected or recognized to cause degradation. Such results are not without value—on the contrary they already give pretty strong pointers towards the form of the final solution—but it is hoped soon to be able to repeat and extend the work on more up-to-date preparations.

Powders of yeast nucleic acid were examined from various sources. None gave a viscous solution, and the diffraction pattern was always either of the amorphous type or at least showed little evidence of crystallinity. Pl. 1, fig. 3, obtained with an Eastman Kodak preparation, was one of the best defined, and the feature to notice is the quite strong reflexion at about 3½ A. We received from Drs Stanley and Loring two early preparations of the nucleic acid from tobacco mosaic virus: one gave an almost amorphous pattern very similar to that given by a yeast nucleic acid preparation sent by Prof. Caspersson, while the other gave the relatively sharp ring pattern illustrated in the right half of Pl. 2, fig. 4. In this latter pattern there are to be seen about a dozen rings altogether, but again it should be noticed that the strongest are at 3·3 and 3·5 A. Then the striking result was found that practically the same ring pattern was given by a preparation,

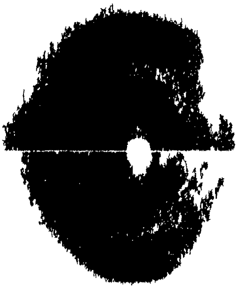
PLATE 1



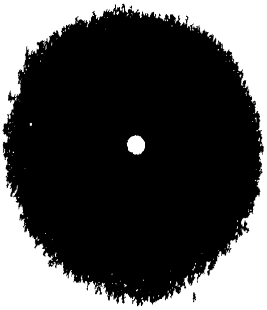
1



2*a*



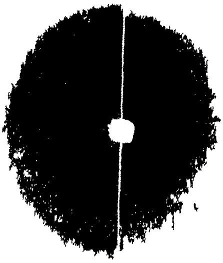
2*b*



3

For explanation see p. 76

PLATE 2



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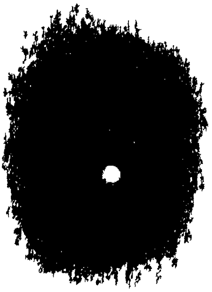

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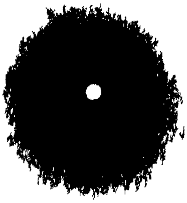

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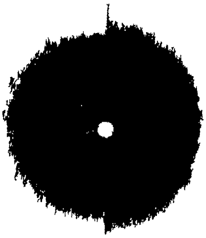

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For explanation see p. 76

sent by Profs. Hammersten and Caspersson, of the pancreas polynucleotide, which is said to resemble closely the nucleic acid from yeast: the comparison with the *tmv* nucleic acid preparation is shown in Pl. 2, fig. 4.

On the whole, considering these similarities, the repeated occurrence of a prominent reflexion at about 3½ A., and the common high density, these experiments leave the reasonable impression that nucleic acids of the ribose type also are built up as columns of nucleotides, and that their main outlines of structure are similar to one another and to that of Na thymonucleate. We shall see below that this impression is very much strengthened, if not actually confirmed, by X-ray photographs of a recent, and highly polymerized, preparation of the sodium salt of *tmv* nucleic acid; but before we go on to that it is worth while recording the results of a few more studies with the earlier preparations.

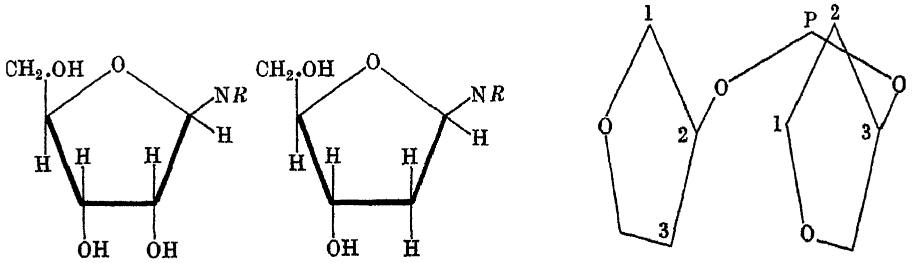
One of these was of what may be called the 'glassy' state that we observed on wetting the powder (as received) of yeast nucleic acid or pancreas polynucleotide with a little water on a microscope slide and allowing the gluey mass to dry. Both preparations made in this way gave a diffraction pattern that is typical of the liquid or glassy state in that there is a clear space round the centre of the photograph (see Pl. 2, fig. 5). It is difficult at present to be sure of the correct interpretation of such photographs in the case of molecules of the complexity of the nucleic acids, but it is a plausible assumption that the position of the boundary of the clear space is a measure of the largest frequent distance of separation of molecules or aggregates. The clear space stops at about 16 A. in the pattern from the yeast glass, and at about 12 A. in the pattern from the pancreas glass (incidentally, we failed to obtain the glass type of pattern with the virus acid); and the interesting point here is that this looks like another argument in favour of a piling of *single* nucleotides; in other words, it seems to be another argument against any idea that may still be current that a primary unit of the nucleic acids is a linked set of four nucleotides lying in a plane. Such a unit would be some 40 A. across, and we should expect the limit of the clear space to be appreciably nearer the centre of the photograph.

Another interesting effect was observed when a mass of yeast nucleic acid powder was dropped into hot water. It developed a consistency something like that of chewing gum, so that it could be rolled out into strips. An X-ray photograph showed then the faint beginnings of orientation—in fact, this was our only success of the kind with acids of the ribose type. Even so, the effect was too feeble to interpret, but it deserves further investigation.

Attempts were also made to obtain fibre photographs of thymonucleic *acid*, specimens of which were prepared by Prof. Gulland and Dr Bailey. Dr Bailey's preparation was actually fibrous in appearance, but it turned out to be a pseudomorph after all, giving only an unsharp powder photograph from which it was difficult to deduce anything new to add to the clues we were looking for. A specimen of barium thymate, sent by Prof. Gulland, did however provide a photograph that appears to throw a certain amount of new light on the problem: it is shown in Pl. 2, fig. 6. There are two rings, one at 11·5 A. and the other at 6·8 A., and it is noteworthy that the latter spacing is twice the internucleotide spacing arrived at from the study of Na thymonucleate. Since thymic acid is produced by removing the purine bases from thymonucleic acid by partial hydrolysis, this observation is readily explained if purine and pyrimidine bases occur alternately along the thymonucleic acid column.

Quite recently we have taken up these studies again with a resumed attack on the structure of the nucleic acid from tobacco mosaic virus, and this time with the product of high molecular weight prepared as described by Cohen & Stanley (1942). Only a tiny amount of the sodium salt has been available as yet—it was kindly prepared by Dr Bailey—but following our usual technique it has been possible to make small areas of thin film by drying the viscous aqueous solution on a glass plate. With many elongated molecules, e.g. gelatin, myosin, and Na thymonucleate, this procedure leaves them lying roughly parallel to the plane of the film, and an X-ray photograph taken with the beam parallel to this plane gives then an approximate fibre pattern. Considering the high viscosity of the new preparation we fully expected to obtain a similar result again, but unfortunately it did not happen: we obtained only a powder photograph. This powder photograph is illustrated in the right half of Pl. 2, fig. 7, the left half representing a corresponding powder photograph of Na thymonucleate. The two ring patterns are not identical—one would not expect that—but it will be seen that the resemblance is so close that in their broader features, at least, the two structures must be very much alike. With the experience gained from these preliminary trials we hope soon to continue the investigation on a new purer preparation, but already I think it may be accepted that there can hardly be any outstanding difference in the way the two types of nucleic acid are built up.

I am indebted to Dr Davies for examining this finding with the aid of atomic models. In the first place, it is clear that a similar superposition of nucleotides can be accomplished using either a ribose or a desoxyribose sugar, because the additional hydroxyl group is found on the periphery of the column. The more important question concerns the nature of the linkage between successive nucleotides, and on this point the evidence of the model is rather arresting. It has been suggested (see Greenstein, 1944, pp. 216–17), for instance, that in the ribose type of nucleic acid the linkage is between C(2) and C(3), but if that were so there would arise the situation depicted diagrammatically in Text-fig. 1. The C-O bonds from C(2) and C(3) will be inclined at about the same angle to the plane of the sugar ring, and therefore the distance between successive nucleotides is defined effectively by the sum of the projections of the two O-P bonds, each $1{\cdot}6_7$ A. long. Taking the O-P-O angle to be of the order of 100°, the sum of these projections comes to little more than 2½ A., a distance of approach that is far too small. One must be chary at this stage of laying down the law about almost anything pertaining to the detailed structure of the nucleic acids, but certainly if the nucleotides in the *tmv* acid lie parallel and directly on top of one another, as suggested by the comparison with Na thymonucleate shown in Pl. 2, fig. 7, it seems impossible for the linkage to be between C(2) and C(3) unless the bond angle subtended at the phosphorus atom becomes 180°, for which there is no evidence from other phosphorus compounds. If, of course, the nucleotides do not lie directly above one another, then the argument does not necessarily apply.



d-ribofuranose 2-desoxyribofuranose

Text-fig. 1.

Judging by the scale model constructed to test the proposed packing of nucleotides in Na thymonucleate, the only way of building such a column is to make use of the —CH_2—OH side chain. Thus present indications are that the main internucleotide linkage in the nucleic acid from tobacco mosaic virus is between C(5) and C(3) or C(2), with slight odds in favour of C(3).

I should like to express my indebtedness to Dr F. O. Bell and Mr L. C. Spark for the X-ray photography, to Dr K. Bailey for unfailing biochemical help, to Profs. Gulland, Caspersson, and Hammersten, and Drs Stanley and Loring, and Mrs Beale for supplying me with specimens, and to Dr M. M. Davies for constructing atomic models. The investigation was made possible by the financial support of the Rockefeller Foundation.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. X-ray fibre photograph of sodium thymonucleate.
Fig. 2. *a*, comparison between the left half of the Na thymonucleate fibre diagram and the right half of the clupein thymonucleate fibre diagram. *b*, comparison between the top half of the Na thymonucleate fibre diagram and the bottom half of the clupein thymonucleate fibre diagram.
Fig. 3. X-ray powder photograph of a preparation of yeast nucleic acid (Kodak).

PLATE 2

Fig. 4. X-ray powder comparison photographs of an early preparation of tobacco mosaic virus nucleic acid (right) and a preparation of pancreas polynucleotide (left).
Fig. 5. X-ray photograph of yeast nucleic acid 'glass'.
Fig. 6. X-ray powder photograph of barium thymate.
Fig. 7. Comparison between the X-ray powder photographs of Na thymonucleate (left) and a high molecular weight preparation of the sodium salt of the nucleic acid from tobacco mosaic virus (right).

THE DISTRIBUTION OF NUCLEIC ACIDS IN TISSUES

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I. THE TOTAL NUCLEIC ACID CONTENT

It is theoretically possible to determine the nucleic acid content of a tissue from estimations of (*a*) phosphorus, (*b*) purine and (*c*) sugar. The last method enables a differentiation to be made between the two types of nucleic acid; the first two supply a figure for the sum of the two types. In practice purine estimations tend to be inconvenient and have been employed by only a few workers, e.g. Alders (1927), and Robertson & Dawbarn (1929). Most authors, therefore, have relied on phosphorus estimations.

Table 1. *Nucleoprotein phosphorus concentration in the tissues of the adult and embryo* (9–10 *week*) *sheep* (Davidson & Waymouth, 1944*b*)

	mg./100 g.			
	Adult		Embryo	
	Fresh tissue	Dry tissue	Fresh tissue	Dry tissue
Liver	70	231	103	592
Lung	88	419	84	763
Gut	110	559	78	673
Heart	29	129	64	492
Spleen	129	566	109	642
Kidney (cortex)	80	421	85	654
Kidney (medulla)	47	324	34	354
Brain (grey matter)	18	114	24	324
Brain (white matter)	53	206	24	253
Muscle	19	75	47	373
Cartilage	21	53	43	199
Thyroid	25	102	78	394
Thymus	—	—	170	1080
Skin	38	107	56	524

The phosphorus compounds in tissues can be divided into three main groups: (*a*) acid-soluble P, (*b*) lipid P, and (*c*) residual or protein-bound P. When a tissue had been exhaustively extracted with trichloracetic and with lipid solvents to remove (*a*) and (*b*) the phosphorus which remains includes nucleic acid P and phosphoprotein P. The content of the latter in most animal tissues, with the exception of materials such as egg yolk and milk, is negligible (Euler & Schmidt, 1934; Davidson & Waymouth, 1944*b*; Schmidt & Tannhauser, 1945), and the assumption is usually made, with some justification, that estimation of residual phosphorus gives a measure of the nucleic acid content of a tissue.

The results obtained by various authors for the nucleic acid content of different tissues from different species have been tabulated in the review of Davidson & Waymouth (1944*a*). The species for which most data are available is the sheep and figures for the nucleic acid phosphorus (nucleoprotein phosphorus, N.P.P.) of adult and embryo-sheep tissues are given in Table 1. As might be expected, the nucleic acid concentration is high in richly cellular tissues such as spleen and thymus and low in tissues such as cartilage and muscle. Comparison of the figures for embryonic and for adult tissues show that, in general, the former are richer in nucleic acid than the latter, and this difference is most marked when the comparison is made on dry tissue. The much higher water content of the embryonic material makes it desirable that the tissues should be compared both on a wet-weight and on a dry-weight basis. It should be emphasized that the figures shown represent the nucleic acid *concentration* in different *tissues* and do not enable us without further data to make comparisons between the nucleic acid contents of the *cells* of different tissues.

II. THE RELATIVE AMOUNTS OF THE TWO TYPES OF NUCLEIC ACID

Animal and plant tissues contain both pentose nucleic acid and deoxypentose nucleic acid (thymonucleic acid), the former mainly in the cytoplasm and the latter in the nucleus. Pentose nucleic acids have been isolated from beef pancreas (Jorpes, 1928, 1934), from sheep liver (Davidson & Waymouth, 1944*c*), from dog liver, intestine and kidney (Brues, Tracy & Cohn, 1944) and from the larvae of the blow-fly, *Calliphora erythrocephala* (Khouvine & Grégoire, 1943; Grégoire, 1945). Although the sugar has been conclusively proved to be *d*-ribose only in the case of the pentose nucleic acids from yeast (Gulland & Barker, 1943) and from the liver (Davidson & Waymouth, 1944*c*), the term ribonucleic acid is generally applied to the pentose nucleic acids from all these different sources. In so far as they have been characterized, the ribonucleic acids from liver and *Calliphora* larvae appear to be very similar to yeast ribonucleic acid in content of purine and easily hydrolysable phosphorus and to differ slightly in these respects from the nucleic acid of the pancreas.

Estimations of the concentration of the two types of nucleic acid in tissues have been made by several different methods. Jorpes (1928) and Brachet (1941 *a*, *b*) have estimated the pentose content of fresh tissues as a measure of the ribonucleic acid. Such estimations, however, usually depend on the liberation of furfural which is colorimetrically determined, and are subject to interference by a number of factors, e.g. the uronic acids of mucopolysaccharides. Davidson & Waymouth (1944*b*, *d*) extracted acid-soluble material and lipins from sheep tissues, removed nucleic acids from the residue with 10% sodium chloride and precipitated them as lanthanum salts which were then used for pentose and deoxypentose estimations. In liver tissue pentose and deoxypentose estimations were also carried out directly on the extracted tissue powder and the results expressed in terms of nucleic acid phosphorus. Schmidt & Tannhauser (1945), who have objected to this procedure on the grounds that the relationship between pentose and phosphorus may vary from one nucleic acid to another, have overlooked the fact that Davidson & Waymouth (1944*d*) used pure liver ribonucleic acid as the standard in all their estimations of the ribonucleic acid content of liver tissue under various conditions. The relationship of pentose to phosphorus is the same in liver ribonucleic acid as in yeast ribonucleic acid.

Schneider (1945*a*, *b*) extracted acid-soluble and lipid P and then treated the residue with hot trichloracetic acid which split off the nucleic acids from the residual protein. Pentose and deoxypentose were then estimated in the acid solution.

Schmidt & Tannhauser (1945) made use of an entirely different principle. After extraction of acid-soluble and lipid P, the residue was treated with *N* potassium hydroxide at 37° for 15 hr. The alkali dissolved the residue completely, split off inorganic phosphate from phosphoproteins and hydrolysed the ribonucleic acid into the constituent acid-soluble nucleotides while deoxyribonucleic acid was unaffected. When the solution was acidified, deoxyribonucleic acid was precipitated along with the protein. Phosphorus estimations on the acid supernatant and on the original alkaline digest could thus be used to determine total nucleic acid, ribonucleic acid (and phosphoprotein) and, by difference, deoxyribonucleic acid.

Some of the results of estimations on liver tissue by these different methods are shown in Table 2. The figures obtained by various authors show fairly good agreement and the striking fact emerges that liver tissue contains about four times as much ribonucleic acid as deoxyribonucleic acid.

Estimations of the amounts of the two types of nucleic acids in different tissues have been made by only a few authors and such results as are available from the literature have been collected into Table 3. Again there is fairly good agreement between the results obtained by different workers, and there appears to be no great species variation. The ratio of ribonucleic acid to deoxyribonucleic acid does, however, vary widely from tissue to tissue. In tissues such as liver where the cytoplasmic volume is high, relative to the nuclear volume, the ratio is high, whereas in tissues with a high nuclear volume, e.g. spleen or thymus, the ratio is low and deoxyribonucleic acid predominates. In isolated nuclei the ratio is of course very low, but even nuclei do appear to contain small amounts of ribonucleic acid. Some of this ribonucleic acid no doubt comes from the nucleoli (*vide infra*), and some may be derived from cytoplasmic residues which are not removed in the isolation of the nuclei for analysis. The histochemical experiments of Brachet (1941*b*) lend support to the view that nuclear material contains small amounts of ribonucleic acid.

If the ratios shown in Table 3 are examined in the light of the total nucleic acid content of the different tissues as shown in Table 1, it will be obvious that the concentration of ribonucleic acid is low in tissues such as muscle, thyroid, heart, etc., and is high in pancreas, liver and gut and in the embryo. Such tissues are actively engaged in the process of protein synthesis. Caspersson and his colleagues (cf. Caspersson, 1941), on the basis of ultra-violet absorption measurements, have concluded that a high ribonucleic acid content is characteristic of cells in which protein synthesis is vigorous for purposes of growth or of secretion.

Table 2. *Nucleic acid content of liver as estimated by different methods*

The results are expressed in terms of ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) in mg./100 g. fresh tissue.

RNAP	DNAP	Ratio $\frac{\text{RNAP}}{\text{DNAP}}$	Species	Author
101	23·6	4·3	Rat (fed)	Davidson & Waymouth (1944*d*)
87	26	3·3	Rat	Schmidt & Tannhauser (1945)
102	22·5	4·5	Rat	,, ,,
79	21	3·9	Rat	Calculated from Schneider (1945*a*)
70	34	2·1	Ox	Calculated from Jorpes (1928)

III. THE DISTRIBUTION OF NUCLEIC ACIDS IN THE CELL

It is now generally accepted that ribonucleic acid is found mainly in the cell cytoplasm while deoxyribonucleic acid occurs exclusively in the nucleus. Behrens (1938) obtained evidence for the presence of ribonucleic acid in cytoplasmic material from which the nuclei had been separated. In the cytoplasm of rapidly growing plant and animal cells Caspersson & Schultz (1940) found material which absorbed ultra-violet light strongly at 260 mμ and gave a negative Feulgen reaction, a positive phosphorus test on micro-incineration, and a strongly positive orcinol test for pentoses. The histochemical test of Brachet (1941*b*), which consists in the removal of basophilic material from tissue sections by treatment with ribonuclease, has

Table 3. *Ratio of ribonucleic acid to deoxyribonucleic acid in tissues (in terms of nucleic acid* P)

Tissue	Ratio	Species	Author
Liver	3·9	Rat	4
	3·3–4·5	Rat	5
	4·3	Rat (fed)	1
	3·1	Rat (fasted)	1
	3·5	Sheep (adult)	2
	2·6	Sheep (embryo)	2
	2·1	Ox	6
	3·0	Rabbit	6
Lung	0·4	Mouse	4
	0·9	Sheep (adult)	2
	0·7	Sheep (embryo)	2
Heart	2·5	Mouse	3
	2·9	Rat	3
	3·6	Sheep (adult)	2
	2·4	Sheep (embryo)	2
	3·1	Chick (embryo)	3
Spleen	1·3	Rat	5
	0·5	Ox	6
	0·5	Sheep (adult)	2
	0·6	Sheep (embryo)	2
Kidney	1·4	Rat	5
	2·4	Rat	3
	1·8	Sheep (adult)	2
	0·8	Sheep (embryo)	2
Brain	1·5	Rat	4
	2·2	Rat	5
	2·1	Sheep (adult)	2
	2·3	Sheep (embryo)	2
Muscle	3·6	Sheep (adult)	2
	1·4	Sheep (embryo)	2
Skin	1·9	Sheep (adult)	2
	1·0	Sheep (embryo)	2
Testis	2·6	Sheep (adult)	2
Ovary	1·3	Sheep (adult)	2
Placenta	2·0	Sheep	2
Thymus	0·3	Calf	5
	0·2	Calf	6
	0·2	Sheep	2
Nuclei	0·2	Embryo sheep liver	2
	0·1	Frog gut	7
Pancreas	15·4	Ox	5
	5·4	Ox	6
	8·0	Ox	2
Parotid	0·6	Ox	6
Stomach mucosa	5·3	Pig	6
Gut	1·0	Sheep (adult)	2
	1·1	Sheep (embryo)	2

(1) Davidson, J. N. & Waymouth, C. (1944*d*).
(2) Davidson, J. N. & Waymouth, C. (1944*b*).
(3) Davidson, J. N. & Waymouth, C. (1946).
(4) Calculated from Schneider, W. C. (1945*a*, *b*).
(5) Schmidt, G. & Tannhauser, S. J. (1945).
(6) Calculated from Jorpes, E. (1928).
(7) Brachet, J. (1941*a*, *b*).

also been used to demonstrate the presence of ribonucleic acid in cell cytoplasm (Brachet, 1941*b*; Biesele, Poyner & Painter, 1942; Gersh & Bodian, 1943; Davidson & Waymouth, 1944*e*; Dempsey & Wislocki, 1945). Much of this cytoplasmic ribonucleic acid is present in the form of particles or granules (mitochondria; large particles, secretory granules, zymogen granules; small particles, microsomes; cf. Bensley, 1942; Claude, 1943*a*, *b*, 1944), and it has been stated (Brachet & Chantrenne, 1942; Jeener & Brachet, 1941, 1942) that in the adult cell nearly all the ribonucleic acid is present in such particulate, and therefore sedimentable, form, while in rapidly proliferating cells such as those of the embryo, the particles account for only 20–40% of the total ribonucleic acid content. The remainder occurs as 'free' ribonucleic acid which does not sediment out in the ultra-centrifuge.

In the liver cell the large particles can be demonstrated in the ultra-violet microscope as strongly absorbing masses in the cytoplasm (Davidson & Waymouth, 1946). Treatment with ribonuclease removes the absorbing material.

The distribution of nucleic acids within the nucleus is more complex. While there is little doubt that deoxyribonucleic acid is confined to the nucleus, its disposition within the nucleus and its relationship to the other nuclear constituents, especially the chromosomin of Stedman, are less clear. The position of the nucleolus, however, is more definite. There is good evidence that in many cells the nucleolus contains ribonucleic acid. It gives a negative Feulgen reaction; it absorbs ultra-violet light strongly at 260 mμ and its intensity of absorption (as well as its basophilic properties) is reduced by treatment with ribonuclease; it gives positive histochemical tests for pentoses. In certain types of cell, e.g. nerve cells and white blood cells, it may be surrounded by a ring of Feulgen-positive material termed the 'nucleolar associated chromatin' which may eventually form the chromocentre (Caspersson & Thorell, 1941; Thorell, 1944; Hydén, 1943; Hamberger & Hydén, 1945). In the rat-liver cell the central portion of the nucleolus absorbs ultra-violet light less strongly after treatment with ribonuclease, while the peripheral portion, which gives a stronger Feulgen reaction than the central region, is unchanged by such treatment (Davidson & Waymouth, 1946). There is thus evidence that in the nucleolus of the rat-liver cell, ribonucleic acid is concentrated in the central portion and deoxyribonucleic acid in the peripheral region.

IV. THE EFFECTS OF PHYSIOLOGICAL ACTIVITY

During the development of the mammalian embryo, there is a progressive fall in the concentration of nucleic acid in the tissues (Masing, 1911; Le Breton & Schaeffer, 1923; Graff & Barth, 1938; Dumm, 1943). A similar, though less marked, fall appears to accompany increasing age after birth (Andreasen, 1943). To what extent the two different types of nucleic acid are involved in this process is unknown, but it may be significant in this connexion that the relative amounts of the two types is of the same order in the tissues of the embryo sheep as in the corresponding adult tissues (Davidson & Waymouth, 1944*b*).

A progressive fall in the ribonucleic acid content of the cell cytoplasm has been observed during the development of the embryo (Caspersson & Thorell, 1941) and during the maturation of the blood cells (Brachet, 1941*b*; Thorell, 1944). In the development of the sea-urchin egg a decrease occurs in the cytoplasmic ribonucleic acid and is accompanied by a simultaneous rise in nuclear deoxyribonucleic acid, the total nucleic acid content remaining constant (Brachet, 1937). There would thus appear to be a synthesis of deoxyribonucleic acid at the expense of cytoplasmic ribonucleic acid. This would be in accordance with the views of Mitchell (1942, 1943), who maintains that such a process occurs in tumour cells and is inhibited by X- and γ-radiation. Brachet (1945) has pointed out that this process, while easy to demonstrate in the sea-urchin egg, may readily occur in higher organisms, e.g. the chick embryo, where, however, the conversion of ribonucleic acid to deoxyribonucleic acid tends to be masked by the simultaneous synthesis of ribonucleic acid *de novo*. The reverse process, the conversion of deoxyribonucleic acid from chromosomes to ribonucleic acid in cytoplasmic particles, has also been tentatively reported (Pollister & Lavin, 1944).

The distribution of nucleic acids in the tissues may also be influenced by nutritional factors. This has been demonstrated most clearly in liver tissue. During fasting, the total nucleic acid *concentration* in the liver rises owing to loss of glycogen and stored protein, but the absolute amount in the liver falls and this fall is due mainly to loss of ribonucleic acid, the deoxyribonucleic acid remaining almost constant (Davidson & Waymouth, 1944*d*; Davidson, 1945). Fasting is accompanied by a shrinkage in the volume of cytoplasm in the liver cell, by a disappearance of granular material from the cytoplasm and by a loss in phospholipin and protein. The cytoplasmic masses which absorb ultra-violet light tend to disappear on fasting (Davidson & Waymouth, 1946). It appears, therefore, that fasting is accompanied by the disappearance from the cytoplasm of some of the phospholipin-ribonucleoprotein particles which form the 'labile liver cytoplasm' described by Kosterlitz (1944). Similar changes follow the administration of a protein-poor diet (Kosterlitz & Campbell, 1945). A marked fall in the nucleic acid phosphorus content of the lymphoid organs of the rat also follows a period of inanition (Andreasen, 1943).

The presence of ribonucleic acid in the basophilic granules in the anterior pituitary has been demonstrated histochemically by Desclin (1940) and by Dempsey & Wislocki (1945). The amount appears to increase during pregnancy and lactation and after administration of oestrogens and gonadotrophin (Desclin, 1940). Over-stimulation of glandular tissue by electrical or chemical (pilocarpine) methods may result in a fall in ribonucleic acid content (Verne, 1927; Ries, 1935, 1940). A similar fall in ribonucleic acid content is found in the cells of the cochlear ganglion after acoustic stimulation and trauma (Hamberger & Hyden, 1945). The Nissl granules of nerve cells contain ribonucleic acid which disappears during the chromatolysis which follows root section (Gersh & Bodian, 1943).

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BACTERIAL NUCLEIC ACIDS AND NUCLEOPROTEINS

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I. INTRODUCTION

The vast literature of bacteriology contains many references to nucleic acids and nucleoproteins, but the information is very scattered and no systematic approach to the subject has been made. Nucleic acids appear to be present in all bacteria and indeed in all micro-organisms, and usually their identification has depended upon the well-known colorimetric methods so that the question whether or not the bacterial nucleic acids are identical in structure with the better known ribo- and deoxyribo-nucleic acids from plants and animals is still undetermined. It is not the purpose of this account to review the whole of the literature on the subject but rather to give an account of the more important observations in the field and in particular to draw attention to the many problems awaiting investigation. Frequently a brief outline of methods of isolation and properties of products is given for comparison purposes.

In general the bacterial cell, when compared with plant or animal cells, presents the cytologists with much more difficult fields of study owing to the fact that the various parts of the cell are less clearly defined. The question as to the nature of the bacterial nucleus presents a particularly difficult problem which up to the present has not been satisfactorily solved. The literature on this matter has been carefully summarized by Lewis (1941), who divides the various conflicting theories concerning the nucleus of bacteria into the following groups:

(1) The bacteria do not possess a nucleus or its equivalent.

(2) The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm.

(3) The bacterial body is a nucleus devoid of cytoplasm: a naked nucleus or nuclear cell.

(4) The nucleus consists of several chromatin bodies, a chromidial system, scattered throughout the cytoplasm.

(5) The form of the nucleus is not constant throughout the growth cycle. It may occur as a discrete spherical body, an elongated chromatin thread or scattered chromidia depending on the stage of development: a polymorphic nucleus.

(6) The nuclear substance consists of fine particles of chromatin dispersed uniformly in the cytoplasm but is not distinguishable as morphological units: a diffuse nucleus.

(7) The protoplast contains one or more true vesicular nuclei.

(8) The nucleus is an invisible gene string, or a chromatin encrusted gene string analogous to a single chromosome.

Lewis discusses critically the merits and demerits of these diverse views, and although pointing out that no person is yet competent to reach a satisfactory conclusion regarding them, rejects groups 2, 3, 4, 5 and 7 above.

Of these theories that of group 8 appears to be most acceptable to Dubos (1945), who emphasizes some of the difficulties concerned with the bacterial nucleus question. Thus neither staining methods, nuclear division, nor an analysis of the transmissibility of hereditary characters, can be used as evidence of true nuclei. The basophilic dyes are not entirely selective owing to the large amounts of so-called 'reserve' ribonucleic acid in the cytoplasm, ultra-violet absorption methods are not precise because of the presence of ribonucleic acids and coenzymes, while electron microscope photographs do not show anything definite. Results obtained by the Feulgen method for staining deoxyribonucleic acid fails to convince many people because of the necessity for a preliminary acid treatment of the cell. We have recently shown that this acid treatment is necessary in order to convert deoxyribose into ω-hydroxy laevulinic aldehyde ($CH_2OHCOCH_2CH_2CHO$) which is extremely labile and if present in small amounts may be converted into laevulinic acid before reacting with the Feulgen reagent (Stacey, Deriaz, Teece & Wiggins, 1946). The most recent work on the question of the bacterial nucleus, summarized in an addendum to Dubos's book (1945), is that of Robinov, whose demonstration of bacterial chromosomes has opened up new possibilities of great importance.

II. NUCLEIC ACIDS AND NUCLEOPROTEINS FROM VARIOUS BACTERIA

The following account of nucleic acids and nucleoproteins in various bacteria is by no means exhaustive, but one hopes that it will serve to indicate the state of our knowledge on the subject. From the chemist's point of view many of the compounds described cannot be regarded as entities.

Mycobacterium tuberculosis. The existence of nucleic acids in this organism has long been recognized, and it was considered at one time that there was present in the cell a unique type, namely, tuberculinic acid. The high nitrogen content (7·6 %) and the phosphorus content of the cells (0·8 %) were recognized by Schweinitz & Dorset (1895, 1896), while Ruppel (1898) claimed that the organism contained nucleic acid which was bound to a basic protein. He postulated the existence in the cell of a protamine-nucleate. Levene (1901) separated from the cell a mixture of polysaccharide and nucleoprotein by means of extraction with ammonium chloride or with sodium chloride. The fraction containing nucleic acid was precipitated by means of either acetic acid or picric acid. Bendix (1901) first reported the presence of a sugar, probably a pentose, in nucleoprotein fractions from the organism, though later Goris & List (1920) claimed that the sugar constituent was a hexose. The demonstration that tuberculinic acid closely resembled the nucleic acid of the thymus gland was due to the researches of Brown & Johnson (1922, 1923*a*, 1923*b*). These workers obtained the tuberculinic acid by extraction of the dried cells with 1 % sodium hydroxide, and they removed the protein by means of precipitation with picric acid. The nucleic acid precipitated from the supernatant by means of alcohol, contained P, 8·1 % and H, 11·3 %. It was further purified via the copper salt. It gave only low yields of furfural with concentrated hydrochloric acid and appeared to be unique in its lack of uracil, low pentose content and in containing an unusual pyrimidine constituent. Coghill (1926) extracted nucleoprotein fractions by means of alkaline buffers and by electrolytes such as NaCl, NaF, NH_4Cl, etc. The product extracted by NaCl contained 11·3 % of nitrogen, but the nucleic acid constituent was not investigated. Various workers subsequently reported that carbohydrate and protein fractions from the cell contained phosphorus, and Spiegel-Adolf & Seibert (1934) found that a purified tuberculin, precipitated from the culture medium by means of trichloracetic acid, showed an absorption band in the ultra-violet identical with that of nucleic acid. Reprecipitation of the tuberculin with ammonium sulphate removed the nucleic acid and gave a product which showed no absorption band.

Attempts to remove nucleic acid from tuberculin by means of chromatographic absorption methods were not successful (Seibert, Pederson & Tiselius, 1938), but the separation was accomplished by means of electrophoretic methods (Seibert & Watson, 1941). A polysaccharide component was relatively immobile and was easily removed, while at *p*H 5·0 and below, the nucleic acid and protein travelled as a single component. At *p*H values greater than 5·0 the nucleic acid and protein travelled independently, though Seibert (1940), considered that part of the nucleic acid and protein were present as nucleoprotein. The separated nucleic acid was of the deoxyribo-type and was without immunological activity. The active principle in the tuberculin skin test was the protein, since removal from it of the nucleic acid did not impair its activity. This work has been reviewed by Seibert (1944).

Recently (Kent & Stacey, unpublished results) we have shown that deoxyribonucleic acid is associated with some of the fatty components of the cell.

Mycobacterium phlei (timothy grass bacillus). Coghill (1931) has made a study of the nucleic acid of this organism. The defatted cells were ground in a ball mill at room temperature with 20 % sodium hydroxide. After 1 hr. the mass was diluted with water, neutralized (litmus) with acetic acid and centrifuged. The supernatant liquid was treated with colloidal ferric hydroxide and the precipitate filtered off and discarded. The nucleic acid was precipitated from the supernatant liquid by addition of 1 vol. of alcohol containing hydrochloric acid. The precipitate, when dried, was a white powder which gave strong pentose and negative protein tests. Guanine, adenine, cytosine and uracil were identified but no thymine was detected, by colorimetric methods.

Bact. typhimurium (mouse typhoid) (Akari, 1938). The above method of Coghill was used on ether-extracted cells. The protein was removed by dissolving the product in 10 % sodium acetate and then 5 vol. of acetic acid were added. The precipitate was discarded and the filtrate was treated with 10 % cuprous chloride to precipitate copper nucleate. The copper salt was decomposed with acetone containing 5 % hydrochloric acid. The insoluble precipitate after several similar treatments was dissolved in water containing a little sodium hydroxide. It was then centrifuged, acidified with hydrochloric acid (Congo red) and the nucleic acid (total yield 1·6 %) then precipitated by addition of 2 vol. of alcohol. It gave tests for both pentose and deoxypentose and contained nitrogen 14·5 %, phosphorus 9·07 %, ribonucleic acid 14·1 %, deoxyribonucleic 24·8 %. Cytosine, uracil, thymine, guanine and adenine were detected in the product.

B. coli. A nucleic acid of the deoxyribo-type was obtained from this organism by Schaffer (1932).

Azotobacter chroococcum. This organism was shown by Stoklasa (1908) to contain a 'nucleoprotein'.

Corynebacterium diphtheriae. A nucleic acid preparation containing both types was isolated by Coghill (1932).

Streptococcus. The nucleic acids and nucleoproteins of types of this coccus have been studied fairly extensively both from the chemical and immunological aspects. The investigations of Lancefield and of Mudd and his colleagues on these streptococcal constituents are of the highest importance. The serological work will be outlined in § III. Heidelberger & Kendall (1931) were among the first to investigate the streptococcal nucleoproteins. They isolated a series of fractions from *Streptococcus*, some of which were closely related serologically to Lancefield's M proteins. Whole *Streptococcus* cells, defatted by extraction with acetone and ether, were finely ground and then suspended in a buffer at *p*H 4·2 with the object of bringing the acid proteins past their isoelectric points, thus splitting them off from the bases with which they were combined in the living protoplasm. After being washed, the acid-treated cells were extracted with a phosphate buffer (*M*/15) at *p*H 6·5. Further successive extractions were then made at *p*H 8, 9, 10, etc. From the *p*H 6·5 extract there was isolated a nucleoprotein (1·4 g. from 60 l. of culture) which was labile to alkali. (For example, when it was suspended in *N*/200 NaOH and reprecipitated much nucleic acid was split off and there was formed a degraded product having the properties of fractions extracted from the cells at a higher *p*H.) The nucleoprotein had $[\alpha]_D+9{\cdot}5°$; N, 15·4 %; P, 3·8 %; basic ash, 0·9 %. Compared with this a typical fraction extracted at *p*H 8 had $[\alpha]_D-44{\cdot}0°$; N, 14·0 %; P, 1·1 %; ash, 1·3 %. The nucleic acid appeared to be of the ribo-type. It was claimed that the product extracted at *p*H 6·5 was a true nucleoprotein with a readily saponifiable ester linkage on the ground that it gave no precipitate with barium acetate. This claim is not necessarily valid, since some nucleic acid preparations fail to give a precipitate with barium acetate, especially in dilute solution.

Sevag, Lackmann & Smolens (1938) carried out an important and extensive investigation of *Streptococcus* nucleoproteins. Their researches would appear to have been stimulated by the demonstrations that bacteriophage is probably a nucleoprotein (Northrop, 1937), and that Stanley's crystalline tobacco mosaic virus is also a nucleoprotein. They used several methods, as below, for isolating the nucleoproteins. These were serologically active, and the authors pointed out that previous methods usually involved the drastic use of hot acid or alkaline extraction of the cells so that any isolated nucleic acid constituents were considerably degraded and devoid of protein. The methods used, which were improvements on previous methods (Mudd, Czarnetsky, Lackmann & Pettit, 1938; Czarnetsky & Flosdorf, 1937) were: (*a*) disintegration of the cells by the sonic vibration method of Chambers & Flosdorf (1936), followed by extraction with 0·85 % saline; (*b*) the bacterial suspension was dried by the lyophile process (Czarnetsky, 1936) before extraction by the method described below; and (*c*) the low-temperature ball-mill method of Mudd, Shaw, Czarnetsky & Flosdorf (1937) was used to disintegrate the cells.

In all three methods the cells were extracted by 0·85 % saline which was then acidified to *p*H 4·5 and the labile antigen precipitated in 10–20 % yield of the dried organisms. It was purified by reprecipitation methods, and when it was necessary to retain its serological activity it was preferable to dry from the frozen state. A typical nucleoprotein fraction had N, 15·8 %; P, 2·7 %; reducing value, 9–13 %; ash, 2 %. The protein was split off from the nucleoprotein, dissolved in sodium carbonate at *p*H 7, by Sevag's method (1934), in which chloroform was used to form an insoluble complex with the protein and thereby remove it from the solution of nucleic acid. The protein was liberated from the chloroform complex by addition of alcohol, while the main part of the nucleic acid was precipitated from the aqueous solution by acidification to *p*H 5. 700 mg. of nucleoprotein yielded 475 mg. of protein (N, 15·7 %; P, 0·73 %; reducing sugar, 4·7 %) and 153 mg. of nucleic acid (N, 15·8 %; P, 9·67 %; reducing sugar, 14·8 %). The last traces of firmly bound nucleic acid in the protein fraction were removed by dissolving it in dilute NaOH and reprecipitating at *p*H 5·7, then dissolving it at *p*H 2·0 and reprecipitating at *p*H 4·5, the whole process being repeated. The purified protein had *c.* N 14·5 %, and tests for P and reducing value were negative. The nucleic acid was purified further by Sevag's method and by reprecipitation from aqueous solutions by means of 4 vol. of alcohol acidified with HCl. A typical sample had P, 9·25 %; N, 15·9 %; purine N, 10·24 %; reducing sugar, 14·4 %. It gave a positive pentose test and a negative biuret. The same authors (Sevag, Lackmann & Smolens, 1940) estimated the two nucleic acids in whole streptococci by a modification of the Dische method (1930). The deoxyribonucleic acid content was 2–6 %. Zittle (1939, 1942) further examined these streptococcal nucleoproteins. The whole undried organisms, for example, were treated with *N*/20 HCl in 2 % sodium chloride at 56°. A nucleoprotein fraction in 4 % yield was isolated. It was alkali labile and possessed certain type-specific properties. It had a phosphorus content of 4 % and apparently contained ribonucleate only. It was split by means of neutral ammonium sulphate into its nucleic acid and protein constituents, the latter (molecular weight *c.* 40,000) after purification contained arginine but neither tryptophane nor tyrosine. It was essentially free from phosphorus (0·1 %) and its nitrogen content was 14·4 %. The ammonium sulphate method of splitting and fractionating streptococcal nucleoproteins was studied extensively by Heidelberger & Scherp (1939). A wide range of fractions was obtained with $[\alpha]_D$ varying from $-20°$ to $+80°$; N, 15·4–16·6 %; P, 1·8–8·5 %; and it was shown that in some fractions part at least of the nucleic acid was bound merely as a salt. With other fractions barium acetate treatment failed to separate nucleic acid from the nucleoproteins.

Pneumococcus. There are many scattered references to nucleoproteins from pneumococci, the most important developments being given in §§ IV and V. Heidelberger (1925) isolated a number of nucleoprotein fractions from the type III cells, but these fractions were not well defined. Stubb (1929) extracted dried cells with water and then further extracted the cell residues with sodium carbonate. Acidification of the extract with acetic acid gave fractions with a relatively high phosphorus content. Thompson & Dubos (1938) carried out some interesting work on pneumococcal nucleoproteins. They extracted the cells (previously heat killed at *p*H 4·2) with neutral saline at 37°, and they made the important observation that during this extraction the cells changed their Gram-positive character to Gram-negative, a phenomenon they thought to be due to enzyme action. On acidification (*p*H 4·2) of the extract there was obtained nucleoprotein fractions having N, 14·2–15·5 %; P, 3·8–4·4 %. The nucleic acid was entirely of the ribo-type, and the protein constituent was deficient in tyrosine and tryptophane. The nucleic acid was broken down by the Kunitz ribonucleinase (1940) from the pancreas, a reagent which can convert Gram-positive pneumococci into a Gram-negative form. Solutions of the nucleoprotein did not coagulate on being boiled, and it was suggested that the protein was of the histone type.

Recently nucleic acids of both types have been isolated from a number of organisms including pneumococci, *B. subtilis*, *Cl. Welchii*, etc. (Henry, Stacey & Teece, unpublished results). Some of these are mentioned in § V.

III. IMMUNOLOGICAL PROPERTIES OF NUCLEOPROTEINS AND NUCLEIC ACIDS

The classical work of Mrs Lancefield (for review see Lancefield, 1941) on the antigenic structure of haemolytic streptococci has been supplemented in an elegant manner by chemical and physical studies on the streptococcal nucleoproteins carried out by Stuart Mudd and C. A. Zittle and their colleagues. Two types of streptococcal nucleoprotein (the term being used for material containing nucleic acid and protein without recognizing the bond between them) were discovered by Lancefield; one is generally group-specific (the 'P' protein) and the other is type-specific and was termed the 'M-protein'.

Heidelberger & Kendall (1931) had reported that nucleoprotein fractions of a scarlatinal strain of *Streptococcus haemolyticus* reacted with antipneumococcal horse serum, and later Mudd & Wiener (1942) confirmed that haemolytic streptococci belonging to Lancefield's group A contain nucleoproteins closely related to those of *Pneumococcus*. Indeed, it was further shown that in respect of nucleoprotein constituents the haemolytic streptococci belonging to Lancefield's group A are more closely related to pneumococci than to haemolytic streptococci belonging to Lancefield's group B. Two strains of *Streptococcus viridans* appeared to possess unusual nucleoproteins in regard to their serological activity.

The chemical and physical properties of the M substance have been extensively studied, a typical paper being that of Zittle (1942), which describes the separation of the protein and the nucleic acid components of the type-specific M substance as well as some chemical and serological properties of the M substance. The chemical properties were typical of some of the fractions described in § II. Some interesting electrophoretic studies on the M substance were made by Zittle & Seibert (1942). Thus it could be shown that the nucleic acid was free and its electrophoretic mobility was compared with that of yeast and thymus nucleic acid. The M-protein was shown by Wiener, Zittle & Mudd (1942) to be weakly antigenic.

The suggestion has several times been made that nucleic acids themselves have an immunological role; thus Menzel & Heidelberger (1938) noted that nucleic acids from acid-fast bacilli could be precipitated with homologous serum. Heidelberger & Scharp (1939) mentioned the precipitation of streptococcal nucleic acid, though it was likely (Heidelberger, 1940) that in both these cases the nucleic acids were impurities. Pennel (1940) noted that certain nucleic acids from *Brucella* remove antibody from a *Brucella*-specific serum. Winkenwerder, Buell & Howard (1939) described cutaneous reactions with nucleic acid and its decomposition products in certain ragwood pollen-sensitive individuals. A study was made of various types of nucleic acid by Lackmann, Mudd, Sevag, Smolens & Wiener (1941). The samples used were: (*a*) a streptococcal ribonucleic acid (N, 15·5 %; P, 8·87 %); (*b*) a streptococcal nucleic acid containing both ribo- and deoxyribo-types (N, 16·2 %; P, 9·12 %); (*c*) yeast nucleic acid (N, 14·6 %; P, 8·0 %); (*d*) nucleic acid from the thymus gland (not analysed); (*e*) nucleic acid from the tobacco mosaic virus (not analysed); (*f*) nucleic acid from the tubercle bacillus (not analysed); and (*g*) nucleic acid from bull sperm (not analysed). The nucleic acids were set up with rabbit sera against streptococci, pneumococci and acid-precipitated pneumococcal nucleoproteins and with horse sera against streptococci, pneumococci, and several other organisms. Also various hydrolysis products of nucleic acids were tested for their power to inhibit the specific reaction between nucleic acids and horse antipneumococcal sera. The general conclusions were that nucleic acids precipitate specifically with certain antisera—particularly with horse antipneumococcal sera. The specific antibodies appear to be located in the euglobulin fraction of the serum and the specific reaction is very sensitive to *p*H change. The specific reaction can be inhibited by purine nucleosides, purine nucleotides and by purine bases. Pyrimidine bases show weak inhibition only, while pentoses and phosphates give no inhibition.

In his recent book Dubos (1945) hints at a possible antigenic role for nucleic acid when combined with specific capsular polysaccharide. Thus he states that the *Pneumococcus* capsular antigen possesses a complex structure, one part of which is present in all pneumococci while another part, present only in encapsulated cells, varies in composition from one type to another and confers upon each type the immunological specific character of the polysaccharide. He points out that all types of pneumococci, whether encapsulated or not, contain an enzyme capable of inactivating the capsular antigen of all types without destruction of the polysaccharide. This enzyme has an action similar to that of the ribonuclease of animal tissues which also can destroy the activity of the capsular antigen, and decreases the affinity of the cell for basophilic dyes (see § V). Moreover, the capsular antigen is extremely resistant to proteolytic enzymes, so that it may well be that it consists of a ribonucleic acid in combination with a specific polysaccharide.

IV. THE ROLE OF DEOXYRIBONUCLEIC ACID IN THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

One of the most solid achievements in the bacteriology of pneumonia is the characterization of more than forty different types (listed by Boyd, 1943), and the isolation from each of the specific polysaccharide which characterizes each type. It is an interesting fact that these types have never been shown to undergo spontaneous transformation from one type to another, although the reversible conversion of the smooth into the rough form is well known in many types. Griffiths (1928), however, succeeded by cultural methods in transforming an attenuated and non-encapsulated rough (R) variant derived from one specific type into a fully encapsulated and virulent smooth (S) cell of a heterogeneous specific type. This work has been repeated and extended by other bacteriologists, notably Dawson & Sia (1930, 1931). A chemical basis has now been provided for the phenomenon by Avery, McLeod & McCartney (1944), who have demonstrated the essential role played by deoxyribonucleic acid in the transformation. The particular transformation (or transmutation) achieved was the conversion of an unencapsulated type II R *Pneumococcus* into a capsulated type III S form.

The type II R form was obtained as a variant by growing a type II S strain in broth containing type II antipneumococcal rabbit serum for thirty-six serial passages. This R strain by then had lost all the specific and distinguishing characteristics of the parent organism and consisted only of attenuated and now encapsulated R variants. The factor responsible for the conversion of the type II R into the type III S was extracted from encapsulated type III S which had been heat-killed at 65° and thrice washed with saline to remove a large part of the capsular polysaccharide, ribonucleic acid, protein and somatic polysaccharide. The cell residue was then extracted three times with saline containing 0·5 % sodium deoxycholate and the liquid centrifuged. The factor was precipitated from the colourless supernatant liquor by addition of 4 vol. of alcohol. It was a fibrous product which was dissolved in water, deproteinized by Sevag's (1934) method and then reprecipitated by adding 3–4 vol. of alcohol. The last detectable traces of capsular polysaccharide were removed from the factor by incubating it at 37° for 6 hr. with an enzyme preparation capable of hydrolysing the type III polysaccharide, the end-point being determined by failure to react with a patent type III antibody. Finally, the product was deproteinized again and fractionally precipitated with alcohol. 10–25 mg. of substance in the form of fibrous material were obtained from 75 l. of culture. When dissolved in water it lost its transforming power in 2–3 days, but when dissolved in saline and stored at 4° C. or when stored in the frozen state it retained its activity for 2–3 months. It formed highly viscous solutions in water, contained no detectable protein or lipoid, gave a strong Dische test for deoxy-sugars and only a faint pentose test. A typical sample had P, 9·0%; N, 15·4%; and a N/P ratio 1·69. It was unaffected by trypsin or chymotrypsin. It was electrophoretically homogeneous, moving at the same rate as a nucleic acid, it had a molecular weight of 500,000 and an absorption band in the ultra-violet at 2600 A. It seems proven that the substance was a deoxyribonucleic acid of which as small an amount as 0·02–0·003 μg. was sufficient to induce the transformation. There is no doubt that this is an authentic case of a specific mutation caused by a chemical entity, and its importance cannot be over-emphasized.

One is tempted to speculate on the nature of this change, and there are several significant observations which may be of importance. One is that the 'R' cells must be in a so-called state of 'competence' before the transformation can be achieved, and another is that the transforming 'mechanism' must have a connexion or indeed be part of the enzymes which synthesize capsular polysaccharide. More than this, of course, is the fact that the transforming mechanism must form part of the chromosome system.

On one's own view some light may eventually be thrown on the phenomenon by studies on the *in vitro* syntheses of polysaccharide macromolecules of the glycogen and starch types by means of enzymes extracted from animal microbial or plant tissues.

For such syntheses the substrate is known, i.e. glucose-1-phosphate, and it seems reasonably well established that a 'starter', i.e. a substance having the molecular pattern of the final synthetic product, must also be present before synthesis can take place. Thus we have the system:

$$\text{Glucose-1-phosphate} + \underset{\text{(protein)}}{\text{enzyme}} + \underset{\text{(e.g. dextrin)}}{\text{starter}} = \text{synthetic amylose.}$$

Similarly, one has shown that in the *in vitro* enzymic synthesis from sucrose of bacterial dextran, which is indeed a capsular substance in *Leuconostoc mesenteroides*, etc., that a minute trace of dextran itself is necessary to start the synthesis. Thus we have

$$\text{Sucrose} + \underset{\substack{\text{(probably of}\\ \text{nucleoprotein}\\ \text{type)}}}{\text{enzyme}} + \underset{\substack{\text{(trace of}\\ \text{dextran)}}}{\text{starter}} = \underset{\substack{\text{(a poly-}\\ \text{glucose)}}}{\text{synthetic dextran}} + \text{fructose.}$$

The nature of the end-synthetic macromolecule can of course be determined by other factors, particularly in regard to the purity of the enzyme preparation, but on a general analogy with these synthesizing systems one might postulate that in the transformation of types the added type III deoxyribonucleic acid combines in the type R cell with a protein constituent to form a nucleoprotein of the autosynthetic or self-perpetuating type. In the presence of a minute amount of type III polysaccharide to act as a starter the nucleoprotein could function as a synthesizing enzyme and build up the fully encapsulated type III smooth cell. It is realized that every precaution was taken to remove polysaccharide, but the last traces might not be susceptible to enzyme attack if combined with nucleic acid. The work has opened up great fields for investigation.

V. THE GRAM-POSITIVE COMPLEX IN MICRO-ORGANISMS

The Gram method of staining micro-organisms consists in staining heat-fixed smears with an aqueous solution of a dye of the pararosaniline series, mordanting with aqueous iodine, washing with alcohol until no more of the blue dye can be extracted, then counter-staining with a red dye of the acid fuchsin series. Those cells which retain firmly the blue dye are said to be Gram-positive, while those which are decolorized by the alcohol and take up the red dye are said to be Gram-negative. The staining method is of great importance and is used widely by all bacteriologists. Many explanations, both physical and chemical, have been advanced to account for the differential staining (see Henry & Stacey, 1946; Dubos, 1945). It is apparent that there must be some fundamental differences between Gram-positives and Gram-negatives to account for such diverse staining reactions. It was discovered (Henry & Stacey, 1943) that Gram-positive organisms could be rendered Gram-negative by various methods, e.g. by enzymic attack and by chemical extraction methods. A process was developed whereby certain organisms, e.g. *Cl. Welchii*, could be stripped of an essential part of their Gram-positive material and separated into a water-soluble Gram-negative extract and a Gram-negative cytoskeleton. A technique was then developed whereby a constituent of the Gram-negative extract could be plated back on to the cytoskeleton to reconstitute mainly a cell which could once more be stained Gram-positive. The essential factor in the extract which could be replated was the magnesium salt of ribonucleic acid, and its presence at the surface of some Gram-positives, and throughout the cell in others, differentiates them from Gram-negatives.

A typical sample of the magnesium salt had the following analysis: C, 39·0; H, 6·0; N, 13·5; P, 9·0; Mg, 5·2; absorption band at 2600 A.

The method of extracting the cells involved the action of a bile salt, preferably sodium cholate (2% aqueous solution), at 60° in presence of oxygen. Material in the extract was precipitated by alcohol, and careful fractionation was necessary to separate it from polysaccharide and traces of protein. It was shown that in order to replate the Gram-negative cytoskeletons successfully they must be maintained in a reduced condition. The best reagent for this was 1% formaldehyde in 0·8% sodium chloride. It was noted that unless care was taken the cytoskeletons could readily become oxidized and no replating could then be achieved. A point of some importance was the fact that normal Gram-negative organisms could not be plated and rendered Gram-positive by this technique.

Brewer's yeast (*Saccharomyces cerevisiae*) was examined by the extraction technique and with many cells the extraction was essentially complete in 2–3 hr. Under normal conditions, the unautolysed yeast cell was coated with a layer of Gram-positive material which stained blue-black by Gram's method. By cautious extraction followed by Gram-staining, however, it was possible to demonstrate the cytoplasm and a large nucleus. When the extraction was carried out cautiously it was possible to demonstrate the removal of ribonucleic acid in stages. Thus at one stage a nucleated cell with a stippled surface could be seen, then later the stippling disappeared and finally the nucleus, to give a whole Gram-negative cell. The extract contained polysaccharides and magnesium ribonucleate; the latter could be replated back on to the cytoskeletons to restore a Gram-positive nucleus with some surface stippling. The stippling and replating effects could be demonstrated very beautifully by photographs taken in ultra-violet light with the Barnard apparatus. The nucleic acids were not specific since in the form of their magnesium salts they could be plated back on to any receptive cell cytoskeleton whatever their origin, e.g. *Cl. Welchii* magnesium ribonucleate could be plated on to yeast and vice versa.

An interesting connexion between the Gram-positive state and smooth and rough forms of colonies of organisms was demonstrated. Thus when *Streptococcus salivarius* was cultivated in a medium where the magnesium content was reduced to a minimum or where there was sufficient acid production to prevent the formation of magnesium ribonucleate, the cocci became essentially Gram-negative. Furthermore, these Gram-negative forms displayed good evidence of nucleation and when subcultured on agar they gave rise to rough colonies, whereas their normal Gram-positive relatives gave rise to colonies which were smooth. Since smoothness in a colony depends on the formation of relatively large amounts of polysaccharide it is apparent that there is a connexion between the presence in the cell of the nucleic acid of the Gram-positive complex and polysaccharide synthesis.

The cytoskeletons from *Cl. Welchii* and from yeast gave an intense Sakaguchi reaction indicative of the presence of basic protein material and it was considered that the Gram-positive complex (i.e. the dye retaining material) is a high molecular complex formed by the combination of magnesium ribonucleate with a reduced basic protein substrate. A successful search was then made for this protein and for the Gram-positive complex (Henry, Stacey & Teece, 1945), and it was possible to extract the complex which had the essential characteristics of a nucleoprotein. The protein constituent of this appeared to be of a novel type and was not a histone or a protamine. Attempts to extract the basic protein directly from the cell cytoskeleton yielded traces only of proteins with a high basic amino-acid content.

The complex was separated from Gram-positives by an autolytic procedure. The cells were set at 37° in a phosphate buffer at *p*H 8 for a few hours. The liquid was centrifuged and a precipitate thrown down by adjusting the *p*H of the supernatant to 5. The solid was purified by reprecipitation methods and when fixed on a slide stained intensively Gram-positive. A typical product from yeast had N, 14·3%; purine N, 2·9%; P, 2·6%; ribonucleic acid, 25%; deoxyribonucleic acid, 3·5%. Both products dissolved in water at *p*H 7·0, contained Mg and —SH groups. When the nucleoprotein in neutral aqueous solution was heated at 60°, the protein constituent came down in denatured form leaving the nucleic acid in solution. This behaviour closely resembled that of the tobacco mosaic virus ribonucleoprotein (Cohen & Stanley, 1942).

A method, involving the use of ammonium sulphate, was devised for the separation of the nucleoprotein into its protein and nucleic acid constituents. The yeast protein was obtained free from pentose and phosphorus. It had N, 14·3%; arginine, 5·5%; contained —S—S— links, and became denatured on being dried or heated. Neither the nucleic acid nor the protein alone could be stained Gram-positive; when, however, the protein was reduced with formaldehyde and recombined with magnesium ribonucleate at *p*H 5 the resulting complex could again be stained Gram-positive. It was not found possible to prepare protein nucleates which would retain the Gram stain in the true manner. It was apparent that in the bacterial

Gram-positive complex the linkage of the protein with the ribonucleic acid was not of the simple electrovalent type. The mechanism of the dye retention was more fundamental than simple combination of dye with a basophilic salt-like molecule. —SH groups and magnesium ions appear to play an important but undetermined part.

The autolysis procedure in the Gram-negative group did not result in the formation of nucleoproteins. It was possible, however, to extract by means of saline, bile-salt derivatives, etc., both proteins and the two types of nucleic acid from certain Gram-negatives. Moreover, these could be precipitated from aqueous solution at *p*H 5 in the form of protein nucleates. These protein nucleates could not be stained Gram-positive although they had a certain amount of dye-retaining character. There appears to be no doubt that fundamental differences exist between the basic proteins of Gram-positives and Gram-negatives and probably between their mode of combination with the nucleic acids. The ratio of the relative amounts of the two types of nucleic acid differs in some Gram-positives from that in some Gram-negatives. Thus in *Cl. Welchii* and streptococci the ribo/deoxyribo-type is greater than 8/1, while in *Bact. typhosum*, *B. Shigae*, *B. proteus*, etc., it is 1·3/1. This ratio may have some significance, especially in its bearing on the mode of union with the proteins. It is important to discover more of the chemical nature of these proteins in order to throw light on the mechanism of the action of some antibodies. It was possible to show (Henry, Stacey & Teece, unpublished results) that when penicillin in sub-bacteriostatic doses acted upon *Cl. Welchii* and produced long thread-like forms, no change was produced in the total amount of nucleic acid or in the ratio of the two types in the cells. On the other hand, certain of the protein constituents of the cell cytoskeletons were obtained in unusual fibrous form.

These discoveries reveal a new significance regarding the nucleic acids and nucleoproteins of bacteria and together with the modern knowledge available on nucleases a novel approach is available to problems of cell structure.

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A STUDY OF TECHNIQUES FOR THE CYTOCHEMICAL DEMONSTRATION OF NUCLEIC ACIDS AND SOME COMPONENTS OF PROTEINS

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I. INTRODUCTION

The problems of cytochemistry may be divided into three groups. The first group, which must be largely solved before the second two can be studied, involves the determination of the distribution of concentrations of various chemical species in the living cell—both in the normal cell and in the pathological cell. The second group is largely physico-chemical in type: it involves the determination, from the concentrations of molecular species (and catalysts, etc.), of the velocities of chemical reactions proceeding in the cell, and of the mechanisms of storage and mobilization of energy, on the chemical level. The third group, which will need to be developed largely simultaneously with the second group, is a task for the physicist—a quasi-engineering problem—to determine the mechanisms of mobilization and organization of energy on the microscopic and macroscopic scale, the nature of the molecular associations which make possible the super-chemical properties of the cell, and, in short, to provide on the cellular level an explanation of the relationship between morphology and function. At present we are engaged on the first of these tasks.

When we approach this field as chemists there are certain broad classes of substances which we recognize, e.g. fats, proteins, nucleic acids. We usually recognize these classes, not by the properties of molecules as a whole, but by the properties of some of their component parts. For example, the nucleic acids we nowadays usually recognize from the properties of purine and pyrimidine components, or of sugar components. To break down these generic classes into molecular species we need additional tests. Thus the protein class may often be subdivided into enzymes. Viewing the field in this manner, it is plain that the term 'nucleic acid' is generic rather than specific, comparable with the term 'protein'. The chemical facts available suggest that a subdivision of this class may probably be made into ribose and desoxyribose nucleic acids. It is, however, by no means certain that all nucleic acids are of these two types. If we try to define what is meant by a nucleic acid more precisely than this, we get into difficulties. We do not know how far the biochemists' preparations of nucleic acid correspond to those occurring in nature: we know that depolymerization may occur during preparation; we also suspect that polymerization may occur; but of the natural range of polymers we know nothing. The cytochemical investigation of nucleic acid must therefore proceed along the following lines: (1) localization of substances falling into the class of nucleic acids; (2) division of the class into groups characterized by their sugars; (3) classification in terms of purine and pyrimidine components. In addition, it may be necessary to add classifications (4) in terms of phosphate components, and (5) in terms of minor constituents. A classification (6) in terms of molecular weight will certainly be necessary before we can assess the physical potentialities of nucleic acids. Other classifications may be revealed as essential as we learn more.

In making these classifications we shall be largely dependent on chemical techniques: it is a matter of great importance that the standards we adopt in making this analysis shall not be less rigorous than those adopted by the organic chemists. We may be sure the organic chemists have not indulged in unnecessary flights of rigour!

In the light of this, let us consider the merits of current techniques for the cytological study of nucleic acid. The more promising of these are: (1) the Feulgen technique, (2) the use of enzymes, (3) destructive benzoylation, (4) ultra-violet microspectroscopy.

II. THE FEULGEN TECHNIQUE

The principle of this technique is that hydrolysis with *N* HCl will split off purines from nucleic acid, revealing, in the case of desoxy sugars, an aldehyde group which will combine with reduced fuchsin to give a purple colour. If we were certain that the only thing which happens in hydrolysis with *N* HCl were removal of purines the technique would be highly satisfactory. But as Stedman & Stedman (1943) have pointed out, the hydrolysis also causes depolymerization, which may well convert the indiffusible nucleic acid of the fixed preparation into a form which, after linkage with reduced fuchsin, is diffusible. Hence the technique cannot be accepted as revealing the presence of desoxy sugars liberated by mild hydrolysis until we know how far the nucleic acid-fuchsin compound is diffusible. Stacey, Deriaz, Teece & Wiggins (1946) have suggested that the substance which recolorizes reduced fuchsin is not the desoxy sugar, but, in the case of desoxyribose compounds, a derivative, ω-hydroxy laevulinic aldehyde ($HOCH_2.CO.CH_2.CH_2.CHO$). Whilst this substance may well be that which is responsible for the main body of the colour formed when the biochemist carries out the Feulgen reaction in a test-tube, it cannot possibly be responsible for the cytologists' reaction. When the cytologist uses the Feulgen reaction thin sections are hydrolysed in N HCl, and washed thoroughly after hydrolysis; thus all small highly soluble molecules, such as ω-hydroxy laevulinic aldehyde, are removed from the section before it enters reduced fuchsin. On the other hand, all cytologists are aware that excessive hydrolysis results in the non-appearance of the Feulgen colour in the tissue section: this loss of colour is perhaps caused by conversion of a prior hydrolysis product of desoxyribose nucleic acid into such substances as ω-hydroxy laevulinic aldehyde. However, the fact that the chemists have already found highly diffusible and still reactive products formed in mild hydrolysis of nucleic acid must heighten our awareness of the need for further understanding of this technique. If established as correct it would permit of classification of nucleic acids in terms of the sugar moiety of the molecule.

III. THE USE OF ENZYMES

The principle underlying the use of enzymes is that by treating sections, smears or squashes with highly purified enzymes, those components of the cell will be removed which are the specific substrates for the enzymes in question. This presupposes (*a*) that completely pure enzymes can be obtained, and (*b*) that the enzyme can obtain access to the substrate. Supposition (*a*) can never be established, for there is no known way of proving that an enzyme preparation is specific for one substrate or family of substrates. Pirie (1940) has discussed this problem in some detail. On the contrary, there is a good deal of evidence that the so-called purified and crystalline enzymes sometimes used in cytology are not one pure enzyme species. The problem of enzyme access to its substrate (supposition (*b*)) also presents difficulties. A monolayer of protein over nucleic acid would probably suffice to prevent access of enzymes to the nucleic acid. The paradoxical situation therefore arises that, if pure ribonuclease were available, it could destroy a cell organ consisting mainly of protein held together by an almost negligible ribonucleic acid matrix, whereas an organ composed mainly of nucleic acid, but held together by a matrix of protein, could be left intact. It is at this point that one begins to wonder whether the 'successful' use of enzymes in cytology has not depended on the impurity as much as on the purity of the enzymes.

Consequently, promising as this method is in theory, its hazards are so great that I have not thought it profitable to use. The studies of Catcheside and Holmes, reported later in this volume, appear to offer support to this view.

IV. DESTRUCTIVE BENZOYLATION

Mitchell (1942) has pointed out that, whereas tryptophane, tyrosine, histidine, purines and pyrimidines may all react with diazonium hydroxides, only the three former substances are affected by destructive benzoylation. Consequently, if a section is treated with benzoyl chloride, tryptophane, tyrosine and histidine are so chemically changed as to become incapable of combining with diazonium hydroxides. The only remaining major group of components of cells which is known to react with diazonium hydroxides is the purine and pyrimidine group, i.e. components of nucleic acid. Hence treatment with a diazonium hydroxide after benzoylation should give a colour mainly in sites of nucleic acid occurrence. This technique has the great advantage over many others that chemical reagents of a high grade of purity may be employed, and that potentially, the treatments involved should not result in any significant change in solubility of the nucleic acid.

On the other hand, there are also some objections to use of the technique as it stands. First, the chemistry of the reaction with nucleic acid is not clear; there is some evidence (Burian, 1904; Johnson & Clapp, 1908) that nucleosides do not couple with diazonium hydroxides, and this point is not covered by Mitchell's control experiments, which were carried out with free adenine, uracil and guanine, i.e. in the absence of the nucleoside linkage. Secondly, benzoylation involved exposure of the section for up to 12 hr. to $2N$ NaOH, a reagent which could hardly fail to remove material, including nucleic acid, from the section. Thirdly, in my hands the technique has given patchy results, some parts of a section being much more affected by the reagent than were others.

V. ULTRA-VIOLET MICROSPECTROSCOPY

This very powerful technique, for the proper use of which we are indebted to Caspersson, depends upon the difference between the absorption spectrum of proteins on the one hand, and of nucleic acids on the other. To particularize, it depends on the absorbing groups of proteins (tyrosine, histidine, etc.) being different from those of nucleic acid (purines, pyrimidines) and also upon the assumption that practically all the fixable purine and pyrimidine of the cell are in nucleic acids. A further assumption is that the absorption spectra of these compounds are not significantly dependent upon the nature of neighbouring molecules. It is therefore clear that ultra-violet absorption spectra, as used at present, do not give results which permit of an unequivocal conclusion that nucleic acid is present in a certain position in the cell. Before such a conclusion could be derived we should need evidence along the following lines: (*a*) how much the absorption spectra of typical nucleic acids are affected by combination with other cell constituents, particularly proteins: studies with 30–40 widely different proteins would be needed, at least; (*b*) confirmatory cytochemical evidence that the absorbing groups are indeed purine and pyrimidine; (*c*) evidence that the purines and pyrimidines are in nucleosidic linkage, i.e. are not forming part of a compound other than nucleic acid. So far as I am aware the necessary information is not yet available. The absorption in the region of 2600 A. by purines and pyrimidines is so strong as to make errors arising from point (*a*) of minor significance in many instances. But points (*b*) and (*c*) are in urgent need of further attention.

There is one alternative mode of approach with this technique. If we have an overall chemical analysis of the particular cell type which is being studied, so that we know what it contains of protein and nucleic acid, etc., and what the ultra-violet absorption spectra of these components are for the particular case at issue, then obviously from an overall study of the absorption spectrum of the different regions of the cell we may be able to draw up a balance sheet and allocate the substances concerned to the different cell regions and organs. In principle, it might even be possible to assign certain compounds to particular parts of a cell organ. But here again there is an embarrassing lack of experimental evidence. The rudiments of the necessary information are known only within the vertebrates, and even there only for a few organs of a few animals, such as the liver of rats and sheep.

To sum up: the Feulgen technique holds out such great promise that one is tempted to throw overboard one's remaining reservations about it; temptation notwithstanding, this may not be done. The use of enzymes has hazards so great that it is highly improbable that it can ever be raised above the status of a confirmatory technique, and even this hope may prove to be illusory. The use of diazonium hydroxides is very promising but needs drastic overhauling. The ultra-violet absorption technique likewise needs drastic overhauling from the strictly chemical point of view.

In the following sections new studies will be described of the Feulgen technique and of the use of diazonium hydroxides. The points which were particularly kept in mind were: (i) the importance of not removing material from the specimen; (ii) the importance of obtaining indiffusible reaction products; (iii) the importance of defining the chemical specificity of the chemical reactions used. The materials used throughout were (*a*) squashes of *Drosophila* larval salivary glands, and (*b*) sections cut from seven blocks made up as follows: (1) gelatin; (2) gelatin + ovalbumin; (3) gelatin + ovalbumin + desoxyribose (thymus) nucleic acid; (4) gelatin + ovalbumin + ribose (yeast) nucleic acid; (5) gelatin + methaemoglobin; (6) gelatin + methaemoglobin + desoxyribose nucleic acid; (7) gelatin + methaemoglobin + ribose nucleic acid. The blocks were embedded in wax and sectioned by the methods customary in histology, and the sections used to test the proposed reactions upon materials of known composition under experimental conditions similar to those used for the squashes. It would have been preferable to have used many more pure proteins and also nucleic acids from other sources. It would also have been better to use the chromosomin of the Stedmans as an acidic protein, and a histone of nuclear origin instead of globin, but my means did not permit of this. Consequently for this, as well as for other reasons, the studies to be presented must often be regarded as of a preliminary character. Gelatin contains a small amount of histidine, no tyrosine and no tryptophane, ovalbumin more histidine, considerable tyrosine and some tryptophane, and haemoglobin differs from ovalbumin in having a large amount of histidine. Tests involving tryptophane were also carried out with casein, and on the test-tube level. The results obtained give some knowledge of the composition of giant chromosomes after fixation: how far this is the composition of the chromosomes of the living cell remains to be determined.

VI. REACTIONS BASED ON HYDROLYSIS OF NUCLEIC ACID

The Feulgen reaction depends upon hydrolysis with *N* HCl. Stedman & Stedman contend that the Schiff base formed by the reaction of the hydrolysis product with reduced fuchsin (magenta) is diffusible. This was tested by submitting a suspension of desoxyribose (thymus) nucleic acid to hydrolysis at 60° C. by *N* HCl. After 15 min. the suspension was cooled, filtered through glass wool, and poured into a large excess of Feulgen's reagent. After 2 hr. in the dark this solution was deeply coloured, showing that a considerable part of the nucleic acid had been brought into solution by the hydrolysis. This solution, containing the coloured nucleic acid-fuchsin Schiff base, was then diluted, either with more of Feulgen's reagent or with SO_2 water, and used as a stain for chromosomes, after cooling to 0° C. to reduce the small amount of hydrolysis which may occur in Feulgen's reagent.

Although the chromosomes had not been hydrolysed, they picked up the Schiff base vigorously, even from solutions so dilute that the colour would not be observed unless particular care was taken. The chromosomes were stained in a manner qualitatively similar to that observed when Feulgen's technique is used. This is in agreement with Choudhuri's (1943) observations. It is therefore beyond doubt that in Feulgen's technique part of the nucleic acid becomes soluble, and that the soluble material has the power to colour just those parts of the chromosomes which are Feulgen-positive.

But there is, nevertheless, a striking difference between squashes stained by the normal Feulgen method and those stained by the preformed Schiff base. In the former the chromosomes only are stained, and in the latter the cytoplasm also takes up stain, though less vigorously, even from the most dilute solutions. It therefore follows that, in so far as the nucleic acid in the squashes is diffusible in the Feulgen reaction, it must be released either in, or in very close proximity to, the Feulgen-positive bands. One cannot regard these experiments as proving that the nucleic acid is initially in these bands, but they do show conclusively that the nucleic acid is closely associated with, and moves with, the chromosomes. It would be of great interest to put the Feulgen technique on to a quantitative footing, when comparisons with Caspersson's technique could be made on the same chromosomes. If the quantity of purine and pyrimidine indicated by Caspersson's technique before hydrolysis were closely similar to the quantity of desoxy-sugar found in the bands by Feulgen's technique after hydrolysis, the case for the main body of desoxynucleic acid being in the bands would be greatly simplified.*

Another approach is to prepare a coloured derivative of hydrolysed desoxynucleic acid which has different physical properties from those of the fuchsin Schiff base. 2:4-Dinitrophenyl hydrazine is a suitable compound. Whereas the compound with fuchsin should be more soluble in water and less soluble in non-polar solvents than the original nucleic acid, the compound with 2:4-dinitrophenyl hydrazine should be less soluble in water, and more soluble in non-polar solvents. Treatment of squashes with dinitrophenyl hydrazine after 15 min. hydrolysis with N HCl failed to give a significant colour to the chromosomes. But when treated at 0° C. with a saturated solution of dinitrophenyl hydrazine in $2N$ HCl, or with a saturated solution in 75% alcohol which was 0·5 N in HCl, the bands were coloured, though far less deeply than one could wish. To throw more light on this result, the solution obtained by hydrolysing desoxynucleic acid for 15 min. with N HCl at 60° C. was allowed to react with 2:4-dinitrophenyl hydrazine: the solution slowly became cloudy, and after 30 min. at room temperature squashes were placed in the solution after cooling to 0° C. The final concentration of HCl was 0·02 N. After an hour the squashes were stained lightly. The chromosomes were similar to those obtained by the direct treatment with 2:4-dinitrophenyl hydrazine, but, as was the case with the fuchsin compound with hydrolysed nucleic acid, the cytoplasm was also stained. Consequently, the results obtained with 2:4-dinitrophenyl hydrazine may be taken as confirming those given by fuchsin, but not as justifying any further conclusion. It is possible that more information could be obtained by using a more highly coloured hydrazine.

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* The fact that the desoxy-sugar shown by Feulgen's technique is closely associated with the chromosomes, and that by far the greater part of the purine and pyrimidine are present in the bands, as indicated by Caspersson's technique, makes it very tempting to conclude that the sugar must be in the bands combined with the purine and pyrimidine. But Feulgen's technique is qualitative, and there is no satisfactory demonstration that the desoxy-sugar is chemically linked with the purine and pyrimidine radicals present in the bands. Until this proof becomes available judgement must be suspended.

The general conclusion which is forced upon one by the study of experiments of the type just described is that the use of techniques likely to give rise to readily diffusible reaction products should be avoided if completely definitive conclusions are sought.

VII. TESTS BASED ON THE USE OF DIAZONIUM HYDROXIDES

The major tissue components with which diazonium hydroxides may react to give coloured products are histidine, tryptophane, tyrosine, and purines and pyrimidines. The basic principle of the method used in this work was to allow the tissue to react with a reagent which would eliminate the possibility of one or more of these tissue components reacting subsequently with diazonium hydroxides. Then the deficit in colour found on treating with the diazonium hydroxide will be evidence of the localization of the compounds for which the first reagent is specific.

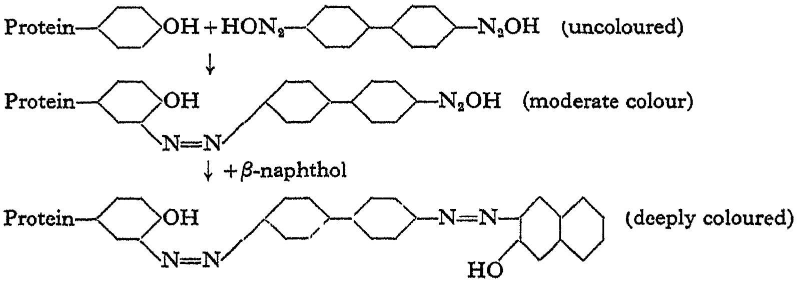

Fig. 1.

The diazonium hydroxides, such as that given by sulphanilic acid, which have been more commonly used in cytology are not as convenient as one could wish. The compounds they give are usually not sufficiently deeply coloured, and decomposition of the diazonium hydroxide during staining may present awkward problems. A suitable compound, giving great flexibility, is tetrazotized benzidine. Benzidine of analytical reagent quality is readily obtainable. When tissue is stained with the tetrazo compound, one end of the molecule forms a diazo linkage with, say, tyrosine, the colour formed being yellowish brown of very moderate intensity. The other end of the molecule retains the reactive —N_2OH group, so that if intensification of colour is required the tissue, after suitable washing, may be allowed to react with excess of a phenol, when an azo linkage is formed by the reactive group with the phenol. The phenols so far found to be most convenient for this purpose are β-naphthol (easily obtained of analytical reagent quality), and H acid (a sulphonated naphthol compound which must be purified by recrystallization). β-Naphthol gives a deep red colour, and H acid a purple. The procedure used was to stain for 15 min. at 0–4° C. with 0·05 % tetrazotized benzidine in veronal buffer, *p*H 9; wash for 1–2 min. with four changes of veronal buffer, followed by 15 min. in 0·05 % H acid or β-naphthol in veronal or sodium bicarbonate. The washing after benzidine must be done with great care, or unlinked tetrazotized benzidine will remain in the tissue, and react with the phenol to give a stain. All the operations must be carried out at 0–4° C. The sections or squashes are then well washed in distilled water, taken through the alcohols and xylols, and mounted in balsam. This procedure has the merit that, providing the tissues are well washed, none of the reagents can give adventitious staining. The hazard of inadequate washing may be checked by using two series of slides, one using β-naphthol and the other using H acid. The compound formed by H acid with tetrazotized benzidine is not readily soluble in alcohol or xylol, and sections stained with it retain their stain when mounted in the usual way. But the compound formed by β-naphthol and tetrazotized benzidine is very soluble in xylol, which extracts all trace of this stain when sections are passed through it. There is, of course, also the hazard that the solubility of the phenol-benzidine-tissue compounds will be significant: but if two phenols such as β-naphthol and H acid are used with two series of slides, a satisfactory control is obtained, for linkage with these two phenols will change the solubility of a tissue compound in diametrically opposite ways.

As Dr Rose has pointed out to me, it is also possible that coloured compounds may be formed by the reaction of diazonium hydroxides with $>NH$ groups to give triazenes. This possibility is eliminated in all cases by washing with cold *N*/100 HCl after treatment with the diazonium hydroxide: this acid washing destroys the triazenes. The formation of triazenes may also be prevented by treatment of material with nitrous acid before use of the diazonium hydroxide: this acts by elimination of —NH_2 groups and nitrosation of $>NH$ groups, so that formation of a triazene is impossible.

Table 1 shows the list of reagents used, and their action upon various groups, as tested upon sections of the seven blocks mentioned earlier in this paper. A positive sign in the table indicates formation of a coloured reaction product between tetrazotized benzidine, and the component in question when the section was first treated with a reagent, then by the diazonium hydroxide. To avoid the use of concentrated sodium hydroxide, benzoylation was performed with 10% benzoyl chloride in dry pyridine. Twenty hours at room temperature gives complete elimination of tyrosine, tryptophane and histidine, with little apparent action upon nucleic acids. Acetic anhydride was also used as a 10% solution in dry pyridine. One hour's treatment at 100° C. gives complete elimination of tyrosine and tryptophane, but the last trace of histidine appears to be difficult to eliminate. Acetic anhydride reduces the intensity of the colour given by the nucleic acids by about 50%. Neither acetic anhydride nor benzoyl chloride would be expected to affect significantly the diffusibility of nucleic acids in the fixed tissues. It is therefore apparent that a tissue component which gives a colour with tetrazotized benzidine, and is resistant to the action of acetic anhydride and benzoyl chloride, is not histidine, tryptophane or tyrosine, and probably contains purine or pyrimidine components. No doubt, however, there are other substances which will react with diazonium hydroxides.

Table 1. *The effect of treatment with various reagents on the reaction of tissue components with tetrazotized benzidine*

+ + + indicates that the component reacts vigorously with the tetrazotized benzidine; + the component reacts less vigorously; − the reactivity is destroyed.

Reagent	Tissue component			
	Tyrosine	Tryptophane	Histidine	Nucleic acid
Benzoyl chloride	+	−	−	+ + +
Acetic anhydride	−	−	−	+
Performic acid	+ + +	−	+ + +	+ + +
Iodine	−	+ + +	+ + +	+ + +
Dinitrofluorobenzene Dinitrochlorobenzene	−	+ + +	+ + +	+ + +

Having regard to the various views which have been put forward as to the protein nature of chromosomes, it seemed desirable to extend this method a little further, so that the sites of tyrosine, histidine and tryptophane may be identified. These techniques, also noted in Table 1, were tested on the seven blocks mentioned previously in this paper, and also on the test-tube scale. Toenies (1942) found that cold performic acid will selectively destroy tryptophane. Performic acid has a strong solvent action on chromosomes, but if brought to *p*H 4·5–5 the solvent action is eliminated, and the ice-cold solution destroys tryptophane without affecting the other groups mentioned in Table 1. Similarly, treatment for 1 hr. with ice-cold iodine in methyl alcohol or in 5% aqueous potassium iodide solution will inhibit the reaction of tyrosine with tetrazotized benzidine. The same effect is achieved by treatment with excess of dinitrofluorobenzene* (Sanger, 1945) or dinitrochlorobenzene (Bost & Nicholson, 1935), as solutions in 90% alcohol saturated with sodium bicarbonate, ice-cold with the former compound and hot with the latter. However, these reactions can hardly be taken as diagnostic for tyrosine—the result would be the same with most phenols.

The dinitrohalogenobenzene compounds can also be used for studying the distribution of other groups. The compound formed with tyrosine is colourless, but yellow compounds are formed with —SH groups and with —NH_2 groups. Hence these can be localized also. The reaction with —SH groups can be eliminated by prior treatment with hydrogen peroxide, and with NH_2 groups by prior treatment with nitrous acid.

Assuming for the moment that the groups in *Drosophila* chromosomes which react with diazonium hydroxides are tyrosine, tryptophane, histidine, and the purine and pyrimidine groups, the results obtained indicate that the bands contain tyrosine, tryptophane, histidine, and purine-pyrimidine groups, and also —NH_2 groups. The interbands contain only traces if any of these groups, except —NH_2 groups, which appear to be present in considerable amounts.

VIII. DISCUSSION

The results described above cannot be regarded as sufficiently defined chemically to give really precise information about the distribution of groups in *Drosophila* chromosomes, but the way does now appear to be relatively clear for obtaining definitive information. The Feulgen technique shows that desoxysugars which are liberated by mild hydrolysis are present in close proximity to the chromosomes. The results with benzoyl chloride and acetic anhydride appear to show that purines and pyrimidines, in so far as they are present in the chromosomes, are mainly confined to the bands. This agrees with the observations made with ultra-violet light. If these two techniques are placed on a quantitative basis and the amount of desoxysugar revealed by the Feulgen reaction shown to correspond to the amount of purine and pyrimidine in the bands, we should be very close to proof that the desoxyribose nucleic acid of the nucleus is actually in the bands.

Clearly we need quantitative specific methods for estimating the amounts of tyrosine, tryptophane, histidine, and the individual purines and pyrimidines. These can probably be determined from the spectra of the azo compounds, and the ultra-violet spectra, provided supplementary techniques are used.* As supplementary techniques must be included the modification or elimination of particular classes of groups by reagents such as those mentioned in Table 1. We particularly need reagents for the individual purines and pyrimidines, which will give known changes in absorption spectra. In Dr Kalckar's paper examples are given of the type which should be useful in cytochemistry. As a simple example we may consider tyrosine: after iodination the pK of ionization of its —OH group is shifted about 3 pK units to the acid side. This shift should permit of its estimation quantitatively by microspectrophotometry, using the ultra-violet region.

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* I am much indebted to Dr A. Sanger for directing my attention to the properties of performic acid and dinitrofluorobenzene: also for the gift of a specimen of the latter compound.

The results given here have an important bearing on the problem of the structure of *Drosophila* salivary gland chromosomes. The interband regions are remarkably deficient in purines and pyrimidines, tryptophane, tyrosine and histidine, but do contain —NH_2 groups.† The bands contain considerable quantities of purine and pyrimidine, tryptophane, tyrosine and histidine. It therefore follows that the interbands cannot consist of typical histones, nor of the chromosomin of the Stedmans, unless these proteins consist of very long polypeptide chains, lengths of which of the order of μ are deficient in histidine, tryptophane and tyrosine, whilst other lengths of similar dimensions are rich in these substances. It has also been noticeable that the amount of purine and pyrimidine found in the chromosomes has been subject to considerable variation. Other workers have recorded the same for desoxy-sugars demonstrated by Feulgen's technique. The protein components of the bands have not varied so widely.

What is clear is that if proteins of histone or chromosomin type are in the chromosomes, then their characteristic aromatic groups are present almost entirely in the bands. Danielli & Catcheside (1945) and Krugelis (1945) have also shown that alkaline phosphatase is present in these bands. The evidence strongly suggests that a gene is an organized system of enzymes. There is a pressing case for the development of more techniques for the cytological localization of enzymes, so that this working hypothesis may be studied further.

In conclusion it must be pointed out that we are still a great distance from achieving that classification of nucleic acids (and proteins) which was indicated as our objective in the introduction to this paper. But it is also clear that there are no serious difficulties in many of the necessary steps. What may be more difficult is the establishment of the exact relationship between the fixed structures and living protoplasm.

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* It must, however, be realized that the nature of the coloured compounds formed when diazonium hydroxides are allowed to react with nucleic acids is not yet established. In fact, all that is known of these compounds is that they are coloured substances formed in sections containing nucleic acid under conditions in which proteins will not give coloured products.

† It is just possible that the interband protein may contain aromatic amino acids combined in a form which makes them unreactive to diazonium hydroxides. This point can be checked by careful comparison of results obtained with ultra-violet light and diazonium hydroxides.

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RIBONUCLEIC ACID AND THE VITAL STAINING OF CYTOPLASMIC VACUOLES IN ANIMAL CELLS

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The accumulation of coloured substances within living cells may involve many different mechanisms. Acid dyes (trypan blue) or negatively charged particles (iron saccharate, chlorophyll) may be stored in the cytoplasm in a granular form; the cytoplasm or the nucleus may be diffusely stained by basic dyes (methylene blue); cell structures such as mitochondria or lipochondria may be selectively stained as by Janus green or neutral red; basic dyes may stain intracytoplasmic particles such as vitelline platelets. Most basic dyes may also be segregated within cytoplasmic vacuoles in living cells; the present paper will deal only with this last type of vital staining.

When the vacuoles which segregate vital dyes belong to the cell structure, they nearly always lie close to the Golgi apparatus. On the other hand, it has long been known that prolonged exposure of living cells to solutions of basic dyes leads to the formation of new vacuoles which take up the dye in a way similar to those of the Golgi region or of the 'vacuome' of Parat.

The chemical nature of the cytoplasmic substances causing accumulation of basic dyes in animal cells has not yet been definitely established. Two main theories were put forward more than 25 years ago. The *lipoid* theory, advocated by Overton and by Nirenstein (1920), considers that the solubility in lipoids of the basic dyes used for vital staining explains why they enter the cell, and become segregated in cytoplasmic structures containing lipoids—for instance, the so-called 'lipochondria' studied recently by Ries in glandular cells. v. Moellendorff (1918) strongly criticized Overton's theory. He argued that the basic dyes which were the most lipo-soluble give diffuse cytoplasmic staining, granules being demonstrated only by weakly lipo-soluble dyes. v. Moellendorff was brought to the conclusion that chemical properties were more important than solubility, and suggested that the cytoplasmic inclusions which were stained had an *acid* character. Kedrowski (1937) stressed the important part which these hypothetical acid substances seemed to play in cell metabolism, by observing the inhibition of the growth of tadpoles living in a 1/2,000,000 solution of neutral red. He suggested in

1941 that the substances stained by neutral red were related to the basophilic proteins of the cytoplasm, and had a complex chemical nature, ribonucleic acid being one of their components. It is worth noting that Zipf (1927) had already reached a similar conclusion by studying the fixation of dyes in perfused tortoise liver: 'the stainability of cells by basic dyes is not related to their lipoids, but depends on their nucleic acid content'.

It may already be pointed out that the two principal theories with which we are concerned are not in so great an opposition as may seem. The lipoid solubility must play an important role in the *penetration* of the dyes into the cell and through the walls of cytoplasmic vacuoles. A chemical combination of the dye and substances within the vacuoles may explain why the dyes *accumulate* in some special regions of the cytoplasm.

In this paper, the mechanism of the formation of new vacuoles under the influence of basic dyes will first be considered, and will illustrate how basic dyes are segregated. The nature of the chemical substances with which they are bound will be discussed. The vacuoles of the Golgi region will then come under consideration, and the conclusions reached by the study of the newly formed vacuoles will be tentatively generalized.

I. THE MECHANISM OF VITAL STAINING OF CYTOPLASMIC VACUOLES

Kedrowski (1934) was the first to consider that the intracellular accumulation of basic dyes ought to be studied in the simplest type of cell. His experiments were made on the red blood cells of the frog. The formation of vacuoles on treatment with neutral red in their almost homogeneous cytoplasm brought strong evidence that basic dyes could be segregated by a cytoplasmic reaction in which preformed cell structures played no part. Kedrowski was also able to show that the vacuolation could be induced separately from the staining of the content of the vacuoles.

The rediscovery of the intense vacuolating effects of ammonium salts as well as various aliphatic and aromatic amines and of piperidine derivatives, observed for the first time by Heinz (1890) and by Guerber (1890) on the same material, led to new investigations on the induced vacuoles of the frog's erythrocytes (Dustin, 1941). When the red blood cells of amphibians—or of fishes, reptiles and birds—come into contact with ammonium salts or amines, their cytoplasm rapidly undergoes spectacular changes. Within a few minutes, numerous small colourless vacuoles appear. They progressively increase in number and size until they occupy a large part of the cytoplasm. These changes may be followed *in vitro* for several days without evidence of haemolysis or other cellular damage; it is a 'vital' phenomenon. In animals injected with ammonium chloride (Heinz's experiment), the vacuolation, which reaches its maximum between 3 and 6 hr. later, gradually decreases over a few days. There is no sign of abnormal haemolysis, and it can thus be deduced that the vacuolation is *reversible.*

The vacuoles so formed have several interesting physical characters. They display a certain individuality, and can be squeezed out of the cytoplasm. They are usually spherical, but may affect elongated shapes. This is indirect evidence that they are limited by a complex membrane, most probably containing lipoids. This conclusion is also substantiated by the fact that the vacuole membrane is impermeable to haemoglobin. The vacuoles may be brought to burst at the surface of the erythrocytes, without producing haemolysis.

The vacuoles are not stained by any histological method, after fixation, and appear to contain a large amount of water. On the other hand, they display an intense affinity for basic dyes and are rapidly and deeply vitally stained.* The staining may lead to the formation of a slight precipitate, deeply stained, which moves about inside the vacuole with rapid Brownian movement, indicating the fluidity of the vacuole content. This precipitate is not formed only by the dye, but consists also of some substance bound to the dye: similar but colourless particles may be met after a prolonged exposure of frog's erythrocytes to ammonium salts. The vitally stained vacuoles are in all respects identical with those which appear in the cytoplasm of the same cells after a prolonged exposure to the basic dyes alone. The experiments of Heinz and Guerber, as well as those of Kedrowski, demonstrate that the basic dyes accumulate in the cytoplasm by segregating some intensely basophilic substance within aqueous vacuoles. The mitochondria are not modified by any of these experiments. The vacuoles contain substances normally present in a diffuse state in the cytoplasm, as may be demonstrated by the following experiments.

When nucleated red blood cells are brought into contact with relatively concentrated solutions of basic dyes (more than 1/10,000), an irreversible precipitation of a basophilic network takes place. This network may easily be fixed; it is the reticulum substance well known in the young red blood cells or 'reticulocytes'. Its precipitation may be produced independently of any vital staining, by allowing wet blood films to dry very slowly (in several hours in a moist chamber). It appears that in the nucleated red blood cells, there nearly always exists some amount of this reticular substance in the cytoplasm. This makes it impossible to prove that it is indispensable for the vacuolation. This proof was brought out by the investigation of the reticulocytes of mammals by the same methods (Dustin, 1942). The evidence is here quite definite: the cytoplasm of reticulocytes has exactly the same properties as that of the nucleated red cells. Its vacuolation can be induced by the same methods, and it segregates basic dyes in newly formed vacuoles—as, in fact, the first authors to study reticulocytes, Israel and Pappenheim, had shown in 1896.* Mammalian blood enables us to reach the following important conclusion: the vacuolation, or the vital staining, is restricted to the reticulocytes. Adult red blood cells—which do not contain any substance precipitated by post-vital staining or by the artifice of 'slow drying' (Gavrilow)—show no modifications in dilute solutions of basic dyes or in solutions of ammonium salts. The vital staining and vacuolation are closely related to the existence of the basophilic material forming the 'reticulum'. This material may be studied histochemically.

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* Most of the blue basic dyes have a red metachromatic colour, and stain the vacuoles metachromatically. It is well known that the more concentrated are the solutions of these dyes, the more red is their colour. Metachromasia is known to have in some cases a histochemical significance (detection of sulphuric esters of high molecular weight). In this type of vital staining, it is a consequence of the high concentration of the dye within the vacuoles (Dustin, 1944).

The reticulocytes of mammalian blood are ideal cells for the understanding of the mechanism of vital staining, because of the absence of nucleus, mitochondria and Golgi vacuoles. More complex cells may now be investigated. There exist several proofs that the properties of reticulocytes are shared by many other cells:

(1) The formation of vitally stained vacuoles in the cytoplasm, outside of the Golgi zone, has repeatedly been described. Any prolonged exposure of cells to non-toxic solutions of basic dyes increases the number of basophilic vacuoles. The significance of this phenomenon has often been discussed, and described as a 'toxic' reaction, or a 'secretory' or 'protective' activity of the cell. The experiments on reticulocytes clearly demonstrate that it is a physico-chemical consequence of the entry into the cytoplasm of a basic substance.

(2) In 1927, Chlopin showed that after prolonged vital staining, the basophilic substances of the cytoplasm, after fixation, formed a network that he considered as a secretion of the cells, induced by the dye and which he named 'crinome'. The crinome is found where the vitally stained vacuoles were in the living cell. After the removal of the vital dye, it stains intensely with all basic dyes. As Kedrowski and Popowa have pointed out, the resemblances between the crinome and the reticulum found after post-vital staining of red blood cells are striking. The crinome phenomenon has been observed by various authors using very different types of cells: it may be considered as a most general reaction of the basophilic substances of the cytoplasm toward vital staining.

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* As explained above, these vacuoles will stain metachromatically: they were described as a 'metachromatic substance' by Cesaris-Demel. In 1925, Jokl claimed to have isolated this substance, identifying it as a phosphatide.

(3) Heinz had noticed only the vacuolation of red blood cells of the frog injected with ammonium salts. The cellular changes are much more widespread, and extensive vacuolation of leucocytes and epithelial cells may be observed (Dustin, 1941). These changes can be reproduced *in vitro*, by placing pieces of tissues in solutions of ammonium salts. The vacuoles stain with basic dyes, especially when they are small. Their situation and form appear similar to the stained vacuoles which are formed after injections of basic dyes or by the direct action of these dyes. Similar vacuolations described by other authors (Lewis, Earle, Monne, Lettré and Albrecht) indicate that this is a very constant property of cytoplasm.

It may be stated in a general way that when basic dyes of the neutral-red type (which do not stain mitochondria) act on the cytoplasm of cells containing basophilic substances in a diffuse state, these will partly become separated in a newly formed liquid phase, and the cytoplasm will be progressively crowded with vacuoles strongly segregating the dye.

II. HISTOCHEMISTRY OF THE SUBSTANCES TAKING PART IN THE VITAL STAINING OF NEWLY FORMED VACUOLES

As explained above, there exists so far no method of studying the chemical nature of the intravacuolar substances. It is necessary to rely on indirect evidence. This evidence is the clearest in the case of the mammalian reticulocytes, the adult red blood cells providing an excellent control. The reticulum material may be easily fixed, after its precipitation by post-vital staining or by 'slow drying'. The fixation (for instance, in absolute alcohol) removes the dye used for the precipitation without altering the properties and the structure of the reticulum. One of its characters, in agreement with v. Moellendorff's theory, is its *acidity*. This may be estimated by using basic dyes in solutions of different *p*H (Pischinger's method for the histological measure of the isoelectric point of proteins). In solutions of *p*H 2 the reticulum stains with basic dyes, but is not stained with acid dyes at any *p*H. There is no indication by this method of an isoelectric point, the substance behaving as an acid. A similar behaviour of nuclear nucleoproteins has been observed by Kelley.

By the ribonuclease method, it is easily demonstrated that the strong basophily of the reticulum disappears rapidly after the action of the enzyme: it may therefore be concluded that the principal histological characters of the reticulum are due to the presence of *ribonucleic acid* derivatives. Other properties studied by Key and others include heat resistance, insolubility in strong acids, insolubility in fat solvents, destruction by 1 % NaOH, and indicate that the reticulum may be of a protein nature.

It is well worth noting that this histochemical result finds ample confirmation in previous chemical researches which have apparently passed unnoticed by most cytologists. In 1911, Masing observed that the nucleic acid phosphorus of rabbit's red blood cells increased when the animals were rendered anaemic by blood loss or haemolysis. In 1930 Kay made a study of lipoid and nucleic acid phosphorus of the reticulocytes of man. The figures for the nucleic acid P of red blood cells run parallel to the percentage of reticulocytes. Kay, being unaware of the importance of cytoplasmic ribonucleic acids, thought that this nucleic acid was of nuclear origin. The reticulocytes provide an instance where the results of vital staining, histochemical methods and chemical analysis are in good agreement. The very small amounts of nucleic acid P detected in human blood when the reticulocyte percentage is low (between 1 and 4 mg. %) indicates that adult red blood cells contain only very small amounts of nucleic acids.

The similarities of the 'crinome' substance and of the reticulum have already been underlined. It is not surprising that histochemical studies give similar results. Kedrowski (1941) demonstrated the presence of phosphorus in the ash of the crinome. He showed that its substance was acid (Pischinger's method), and noted that its basophily disappeared after treatment with ribonuclease. These results have been confirmed (1942), though Pischinger's method failed to show any staining with acid dyes, and therefore brought no evidence of a true isoelectric point (amphibian cells). The relations between the ribonucleoproteins of the cytoplasm, which are known, since the work of Caspersson and Brachet, to be normal constituents of all living cells, and the crinome, have been discussed by Kedrowski: it appears that the basic dyes have modified the cytoplasmic nucleoproteins in a certain way which will be discussed further.

III. THE VACUOLES OF THE GOLGI ZONE

In the light of the most recent researches (Hirsch, Baker, Hibbard), the so-called Golgi apparatus appears as a dynamic region of the cell, where vacuoles staining vitally with basic dyes (Parat's vacuome) are associated with lipoprotein cytoplasmic membranes or structures. In its simplest form these two principal components cannot be separated by ordinary histological methods, and appear as lipoid granules vitally stained by neutral red. These granules are named 'Golgi presubstance' by Hirsch, 'lipochondria' by Ries. The Golgi vacuoles are a region of synthesis and elaboration of secretion products, their lipoid envelope fading away as the synthesis proceeds.

Much stress has been laid on the lipoid content of the Golgi zone, as it is related to the histological demonstration of this specialized cellular region by osmic acid and heavy metal techniques. Though Parat's theory emphasized the importance of the vacuoles, their chemical nature has only been studied quite recently. Baker states that 'the vacuoles do not contain sufficient protein to produce a granule in histological preparations...their substance is fluid, presumably watery'. Hibbard and Lavin have studied the Golgi vacuoles in chicken's gizzard epithelium by means of the quartz microscope, and conclude: 'the Golgi zone does not contain appreciable amounts of nucleoproteins, or nucleic acids...except possibly in greater dilution than in the rest of the cytoplasm'. The fact that secretory granules are synthesized in the vacuoles of the Golgi zone points to a probably very complex chemical composition of those vacuoles. They are known to contain in some cases ascorbic acid, and the recent papers of Emmel, and of Deane & Dempsey, show that they may be, in some cells, a region of important phosphatase activity.

Nevertheless, the constancy of the vital staining of these vacuoles in the most different cells indicates that it is a fundamental property, as the recent work of Worley has stressed. This 'chromophily' may possibly be related to some chemical property similar to that of the newly formed vacuoles we have so far studied.

The red blood cells of lower vertebrates contain a type of Golgi apparatus, which, owing to the simple structure of the surrounding cytoplasm, may be easily investigated by histochemical methods. It consists of a small cluster of vacuoles situated at one pole of the nucleus, and is stained by usual Golgi methods, as well as by vital staining. Moreover, these 'segregation vacuoles' exhibit a marked basophily after fixation (Battacharya & Brambell; Dornesco & Steopoe; Dawson). These vacuoles were studied in the erythrocytes of the Axolotl (*Siredon pisciformis*). They can be easily compared with the newly formed vacuoles, induced in the same cells by any of the methods which have been described. They have in common with these: (1) their appearance after vital staining, (2) their shape, usually spherical, but which may become elongated, (3) their independence of the surrounding cytoplasm, out of which they may be ejected, (4) their metachromatic staining with blue basic dyes, (5) their progressive swelling under the action of ammonium salts, which eventually suppresses all differences between Golgi and newly formed vacuoles. They differ by their basophilic reaction after fixation, though this disappears after the swelling induced by bases. This basophilic material is of the ribonucleoprotein type, as is indicated by the ribonuclease method.

A comparison of the two types of vacuoles in erythrocytes leads to the conclusion that their histological characteristics can be explained by similar chemical properties—lipoid membrane and aqueous content with ribonucleoproteins in solution—the Golgi vacuoles having the greatest amount of lipoids and ribonucleoproteins.

The Golgi apparatus of these red blood cells is of a rather exceptional character, though it closely resembles Ries's lipochondria and Hirsch's 'presubstance'. It remains to be studied how far the vital staining of all Golgi vacuoles may be linked with nucleoproteins of the ribose type. This is strongly suggested by the similarity of the staining properties of all Golgi vacuoles; it must be tested by further histochemical work. This will undoubtedly be made difficult by the minute amounts of nucleic acids which are detected by vital staining. Indirect evidence of the possibility of nucleoproteins playing an important role in the Golgi apparatus physiology will be discussed later.

IV. CYTOPLASMIC AND INTRAVACUOLAR RIBONUCLEOPROTEINS

Any theory linking vital staining with cytoplasmic ribonucleoproteins must explain the following apparent contradiction. As has already been mentioned, the Golgi zone of secreting cells does not contain any ribonucleoproteins detectable by ultra-violet microspectrography. On the other hand, the basal or ergastoplasmic region of protein-synthetizing glandular cells, deeply stained by basic dyes *after fixation*, fails usually to stain vitally with the same dyes. Similarly, the strongly basophilic cytoplasm of the young red blood cells, intensely absorbing ultra-violet light (Caspersson & Thorell), only segregates basic dyes in the vacuoles of the Golgi apparatus (not stained after fixation), and fails to form new vacuoles as the reticulocytes. The definitive erythrocytes of the chick, which easily segregate basic dyes in newly formed vacuoles, on the contrary, have a 'hardly noticeable' ultra-violet absorption.

The explanation of these facts, which were first discussed by Kedrowski, is related to the distinction between the basophily of fixed and of living cytoplasm. It is clearly apparent from the behaviour of the ergastoplasm, for instance, that the histological fixation sets free acid groups which enable the nucleoprotein to combine easily with basic dyes. In the living cell, the acid groups of the nucleoproteins are in a combined form, and the results of the work of Caspersson and his school strongly suggest that basic proteins (containing histidine, arginine and lysine) play a prominent part in these combinations. Thus, in embryonic cells, in strongly basophilic regions of glandular cells, etc., the basophilia may be termed *stable*, as regards vital staining.

The cytoplasm of faintly basophilic cells such as the reticulocytes provides a different example, where the nucleoproteins, though detected by chemical as well as histochemical methods, are hardly visible with the ultra-violet microscope. The amount of basic proteins is also negligible (Caspersson & Thorell). On the other hand, vital staining, or the action of amines, reveals a great cytoplasmic *lability*. The equilibrium of the different phases is easily disturbed, and aqueous vacuoles intensely stained by basic dyes are formed. Evidence has been given that these vacuoles do not appear when there are no more nucleoproteins histochemically detectable. The vacuole formation may be related to hydration of the cytoplasm following the setting free of some of the $—PO_3H$ groups of the nucleoproteins. These free groups combine easily with any basic dye, and the consequence is the formation—in a *living* cell—of nucleoprotein-basic dye compounds similar to those seen in fixed tissues. The alteration of the nucleoprotein structure cannot be very important, as the vacuole formation appears to be reversible. The lability of the nucleoproteins of faintly basophilic cells—or of the less basophilic regions of other cells, and in particular the Golgi zone—explains why minute amounts of nucleoproteins may determine such a striking phenomenon as the intravacuolar accumulation of basic dyes.

We have so far reasoned as if all the ribonucleoproteins took part in vital staining, when they are in a 'labile' form. This is probably only true in the case of very prolonged exposure to basic dyes, amines, etc. In the reticulocytes of mammals, the precipitation of the reticulum often takes place after the basic dye has induced newly formed vacuoles. Even when these vacuoles are of a great size, it appears that a certain amount of basophilic reticulum material is still present in a diffuse state in the surrounding cytoplasm. With blue dyes, such as brilliant cresyl blue, the vacuoles appear purple (metachromatic), the reticulum blue. These pictures of partial precipitation of the reticulum gave rise to Cesaris-Demel's conception, shared by many authors, that there are two different substances, a 'metachromatic' and an 'orthochromatic'. It is most probable that there is nothing other than the ribonucleoproteins, in two different physical states.

This point brings up an important question: is the chemical nature of vacuoles and reticulum (or crinome) identical? We have already mentioned an important difference between these two basophilic structures: the vacuolation exists in living cells and appears to be in some cases reversible, whereas the crinome or reticulum results from an irreversible precipitation—by the basic dyes alone, in the case of the reticulocytes, or by the action of a fixative, in the case of the crinome. The great resistance of the reticulum towards chemical agents is an indication that the nucleoproteins have been chemically altered. Another property confirms this idea. The basophily of the reticulum is much greater than that of the fixed cytoplasm of the same red cells in which the reticulum has not been precipitated (polychromasia).

This may be a simple consequence of an increase in the density of the nucleoproteins, gathered in the network, or more likely an indication that free —PO_3H groups have appeared. Observations on the crinome substance of primitive erythroblasts of tadpoles (Dustin, 1944) indicate similarly that the substance which after fixation lies at the place of the newly formed vacuoles induced by vital staining, reacts more readily with basic dyes, after fixation, than the remaining cytoplasm, which is itself, in the fixed state, strongly basophilic.

This leads us to consider three different states of the ribonucleoproteins in the cytoplasm, as related to vital and post-vital staining. Their properties may be summarized in the following table:

Type of basophilia	Examples	Basophilia of		Histochemistry
		I. Living cells	II. Fixed cells	
Stable	Embryonic cells, ergastoplasm	−	+	Ribonucleoproteins associated with basic proteins
Labile, intravacuolar	Golgi zone, newly formed vacuoles	+	− (Exceptionally +)	Ribonucleoproteins with free acid groups (reversible)
'Crinome'	Precipitation artefact in most basophilic cells	−	+ +	Altered ribonucleoproteins (irreversible)

V. DISCUSSION

All recent biological work emphasizes the importance of the cytoplasmic ribonucleoproteins in cellular physiology. If it is true that these substances play the principal part in the intracellular accumulation of vital basic dyes, it must be true that this in turn affects in some way cell metabolism. It is significant that the work of Kedrowski drew its impetus from the observation that the growth of tadpoles was inhibited by neutral red, and that Kedrowski was led to the conclusion that the substances stained by basic dyes, which he named 'anabolites', played a most important role in constructive metabolism. This conclusion is the more remarkable when one considers that it was drawn before the work of Caspersson and Brachet on ribonucleic acid and protein synthesis.

The reticulocytes give another instance of the metabolic importance of substances stained vitally by basic dyes. It has been known since the publications of Morawitz, Warburg, Masing and Wright, that the respiration of reticulocytes is closely related to their basophilic substance. This disappears while the respiration goes on, and Wright considers that it is consumed by the respiration. The various enzymes of nucleotide structure isolated from red blood cells following the researches of Warburg & Christian are most probably related to the nucleoproteins of the immature red blood cells.*

The work of Grafe (1912) on the action of ammonia and amines on the respiration of the red blood cells of the goose gains interest when the morphological effects of those substances on the ribonucleoproteins are known. The inhibition by ammonia (*N*/35) is reversible and disappears after washing with saline. The inhibition may be very considerable; Grafe gives the following figures: ammonia, 79 %; propylamine, 76 %; methylamine, 84 %; dimethylamine, 82 %. All these substances are known to vacuolate the cytoplasm of reticulocytes (Heinz, Guerber). The action of ammonia has been confirmed by Warburg and by Wright (respiration of bird's blood cells).

Caspersson and Brachet have brought forward important evidence linking nucleoproteins of the ribose type to protein synthesis. On the other hand, the importance of the Golgi zone for the elaboration of secretory granules is well known. If the proposed theory that the vital staining of the vacuoles of the Golgi zone, within which granules or more complex structures such as the acrosome are formed, is related to the presence of ribonucleoproteins, the theory of Caspersson would find new support. It is interesting to consider that there exists a whole cycle indicating close relations between Golgi vacuoles and ribonucleoproteins: the synthesis of the vitelline platelets in the egg and their utilization in the embryo. The synthesis takes place in vacuoles which may be vitally stained. As soon as the digestion of the vitelline platelets begins, it is associated with neutral-red vacuoles. Isolated vitelline platelets of Amphibia may stain vitally, a vacuole being formed at their contact (Jägersten). It is well known that in the embryo, the ribonucleoproteins originate from the vitelline platelets. The vital staining would be one of the first indications of the setting free of the ribonucleoproteins which will later diffuse in the cytoplasm.

In suggesting, as a logical consequence of the results obtained from the experimental study of reticulocytes, that the vital staining of vacuoles with basic dyes is an indication that they contain ribonucleic acid derivatives, it must not be forgotten that these vacuoles have most certainly in many cases a very complex chemical composition. They contain substances in very minute quantities, though these play an active role in cell metabolism (for instance, in the Golgi vacuoles). The quantities of nucleoproteins are so small as to escape detection by methods as sensitive as the ultra-violet microscope. The principal purpose of this paper was therefore to underline how the techniques of vital staining have lost nothing of their interest to-day, and may bring useful data in many problems of cell physiology.

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* Most of the biochemical work does not take in account the differences between reticulocytes and adult red blood cells, the enzymes being isolated from great quantities of red cells, mature and immature.

VI. SUMMARY

1. The mechanism of the formation in living cells of basophilic vacuoles by basic dyes and other basic substances is analysed.

2. The vacuoles contain some of the basophilic material which exists in the cytoplasm; when the cytoplasm is not basophilic (adult red blood cells of mammals), it fails to vacuolate and to be stained vitally.

3. The histochemical study of the substance taking part in vital staining, after its precipitation as the reticulum of reticulocytes or the crinome of other cells, indicates that it contains ribonucleic acid.

4. The Golgi vacuoles of nucleated red blood cells contain ribonucleoproteins.

5. The relations between the basophilia of fixed cytoplasm and the vital staining by basic dyes are discussed.

6. It is suggested that the vital staining of cytoplasmic vacuoles, belonging to the Golgi zone or newly formed, may be always a consequence of the existence of small quantities of ribonucleoproteins within these vacuoles. Some cytophysiological consequences of this theory are considered.

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THE RELATIONS BETWEEN NUCLEIC ACID AND PROTEIN SYNTHESIS

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The study of the chemical composition of the individual cell and of its internal structures involves two principal difficulties, one is the smallness of the objects and the other is that the structure of the cell must not be impaired—preferably one should investigate the cell in its living state. Under such conditions it is, of course, too much to hope for quite general methods; we have to limit ourselves to studying certain substances with specially suitable properties.

The work in our institute is entirely concentrated on the problems of quantitative cytochemistry. The mere localization of a substance is not enough for metabolic studies. Trying more exactly to formulate the conditions such methods must fulfil, in order to make possible the determination of a certain substance in a cell as well as its localization, one comes to the conclusion that the methods should allow (see Caspersson, 1932*b*, 1933*a*, *b*, 1936, 1940*a*, *b*; Hydén, 1943*b*; Norberg, 1942):

(1) The identification of the substance in question within the structure of the cell.

(2) The quantitative determination of the substance.

(3) It must be possible to apply the results directly or indirectly to the living cell itself.

If we want to fulfil all these conditions at the same time large numbers of different procedures are eliminated from the very first, and there remains scarcely any group of procedures other than optical ones. Substances with high relative absorption offer special advantages for quantitative optical work. Because of a number of obvious reasons the technically most easily accessible region extends from near infra-red (about 30,000 A.) through the visible range to the near ultra-violet. In the optical spectrum it ought to be possible to work throughout the whole range with substantially the same arrangements.

For the question of nucleic acid and protein metabolism the ultra-violet region is the most important. About 2000 A. almost all organic substances absorb heavily in amounts present in organic tissues. Between this wavelength and 3500 A. several of the most important cell constituents have characteristic absorption bands. These give an opportunity of studying the distribution of the substances in the cell. An especially advantageous example is given by the nucleic acids—they are at the same time present in high amounts in the cells and have a very high selective absorption at 2600 A., caused by the pyrimidine ring in the bases. This absorption is, in fact, so enormously high that in the concentrations present, for instance, in metaphase chromosomes at 2600 A. it will greatly exceed the absorption of any other cell constituent. With this in mind it is easy to see that in a simple ultra-violet photograph, taken at 2600 A., the nucleic acid-rich particles should appear very dark against a lighter background (Pl. 1, figs. 1, 2, 3) (see Hammarsten, Hammarsten & Caspersson, 1935; Caspersson, 1934, 1936, 1938, 1940*b*).

If we want to identify and especially to determine the amount of substances such as the nucleotides, such a photograph at one wave-length is of no value. We must have *a device for measuring the complete absorption spectrum of the cell structure containing the substance in question* (Caspersson, 1936, 1940*a*).

The measurement of the light-absorption curve in a very small part of a microscopical preparation seemed to be difficult because of the complicated optical conditions in the object and in the compound microscope. We had some years before been interested in the theory of light scattering in colloid solution and made some calculations for white solutions (see Caspersson, 1932*b*, 1933*a*, *b*, 1936). From these calculations it could be seen that it was theoretically possible to create optical conditions so that most of these sources of error would be eliminated: these calculations formed the basis for our further work (see Caspersson, 1936, 1940*b*, *c*, 1941; Brandt, 1941; Brandt & Caspersson, 1941; Engström, 1946; Engström & von Hámos, 1944; Hydén, 1943*a*, *b*; Hydén & Caspersson, 1945; Hydén & Hamberger, 1945; Landström, Wohlfart & Caspersson, 1941; Landström-Hydén, Aquilonius & Caspersson, 1941; Lange, 1944, 1945; Malmgren, Thorell, Bjerkelund & Caspersson, 1945; Norberg, 1942; Santesson & Caspersson, 1942; Schultz, Aquilonius & Caspersson, 1940; Schultz & Caspersson, 1940; Thorell, 1944, 1945; Thorell, Bing & Fagraeus, 1945; Thorell & Caspersson, 1941; Thorell & Wilton, 1945; Thorell & Wising, 1944). In my view it cannot be stressed too much that exact knowledge of the physical background and the conditions for every single measurement are absolutely necessary premises for all microspectrographic work.

The first question is to what extent does the distribution of the intensity in the image given by a microscopic lens correspond to the true distribution of intensity in the object. The theory of the microscope is still incomplete on this point, but we can get very close to the solution and define the optical conditions under which the requirements of this question to face p 128

PLATE I

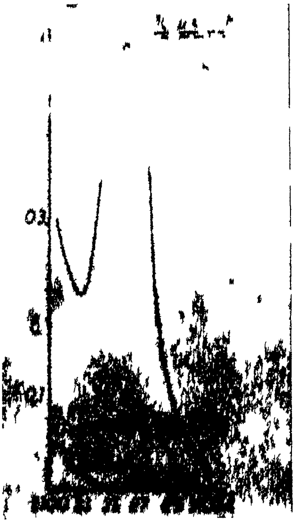


Fig 1

Fig 2

Fig. 3.

For explanation see p. 151

are fulfilled. The calculations show, that if Abbe's sine condition is fulfilled and certain minimal apertures in the lenses are used, one can make real absorption measurements on microscopical objects down to the limit where the laws for geometrical optics are no longer valid.

The second question is the lower limit at which this latter assumption is valid. The theory for that is fairly complex, but using the theory for light dispersion in colloid solutions (see Caspersson, 1932*b*) it was shown that with an error of 5% the absorption measurements can be made of objects down to the size of about four times the wave-length of the light used (that is, in the middle ultra-violet about 0·6 μ), and that there is a continuous change with smaller dimensions giving larger and larger errors until the measurement has no meaning at all. These results are only true if certain conditions are controlled. The aperture is of particular importance. These calculations have one interesting point in that they can be made both for the light scattering in the object and for image formation in the microscope. Both ways give practically the same results (see Caspersson, 1936).

The calculations show that with certain precautions it is theoretically quite possible to measure absorptions down to a certain limit in size, and also that the factors disturbing such measurements can be accounted for.

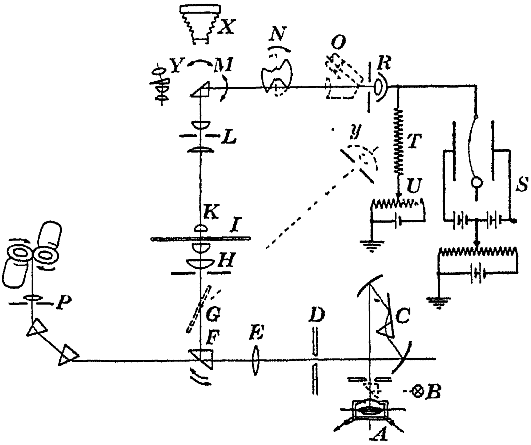
The technique for the measurement offered several difficulties. In these connexions one important factor mentioned earlier complicates the measurement to some extent. It is that a correct absorption spectrum can only be obtained when light bundles with high aperture are used. Such lenses have high magnifications, which makes it necessary to work generally with high magnifications and with the consequent loss of light.

The technically easiest way to make the intensity measurements in the plane of the image is by simple photographic techniques, similar to any of the usual procedures in macrospectrometry. The object is photographed on a plate where at the same time a known light-absorbing arrangement is photographed, such as a wedge, a rotating sector, etc. For our very first experiment we used such a technique (see Caspersson, 1936). As it is impossible to get an absolutely homogeneous distribution of ultra-violet light over a plate in the ultra-violet microscope without very special precautions, we refrained from arrangements of the kind just mentioned and used a more complicated way in which the 'standard system' controlling the development, etc., is exposed separately and with visible light. This method proved very useful for certain questions such as integrating measurements and is still used for simple problems.

Experience showed, however, from the very beginning that no photographic method could give the accuracy needed for highly quantitative work, which would permit detailed analysis of absorption curves. Even in single cases to analyse the absorption curves with any degree of accuracy and at the same time to make corrections for light refraction, etc., it was absolutely necessary to refrain from any use of photographic methods. The error must be less than 1 %, and with a photographic method it is a general experience that you cannot, as a rule, go below 5 %; the special conditions here made it difficult to reach even this value (see Caspersson, 1940*a*). The whole range of applicability of these procedures was thus dependent upon working out a more exact photoelectric procedure. The conditions for the photoelectric measurements could be defined as the following: It must be possible to measure in a continuous spectrum absorptions of areas down to $0{\cdot}1\mu^2$ with an error not greater than 0·1 %. The yield of the available photocells is one limiting factor, and the energy per unit emitting surface in the light source is another. About the photocell there is at present not very much to be done. The sensitivity is limited by the yield of the cell, the number of photoelectrons per thousand photons, and the statistical variation in the electron current, and these conditions are not changed in principle by introducing gas in the cells or arranging multiplicator stages. We might, however, have hopes for the future. If an accuracy of at least 0·1 % is wanted, the statistic variation in the electronic current must be at least 10,000–100,000 times lower than the total current. This gives an average total photocurrent of not less than 10^{-14} amp. We use amplifiers as well as electrometers. The latter are to be preferred because of a number of reasons. One of the more important reasons is their stability at very high sensitivity, which for the Hoffman type can go down to measuring currents of 10^{-17} amp. of 1 sec. duration, that is, a hundred electrons. For the electric measurement we use different electrometers, specially built photocells with very high insulation resistance or multipliers and circuits with automatic correction of variations in the light source.

One limiting factor in these measurements is the specific intensity of the light source. As one can never, of course, by any condensing system get brighter illumination of an object than the specific intensity of the light source, and since the objects are as small as 10^{-7} mm.^{2}, a source has to be found with continuous spectrum and an enormous specific intensity. In 1936 no such source existed for ultra-violet, but the following year we got through the courtesy of Philips laboratories in Eindhoven some of their laboratory models of the now well-known super high-pressure mercury lamps. This gives sufficient light above 3000 A. Below 3000 down to 2300 A. the measurements can just be made. Text-fig. 1 shows the general arrangement of the apparatus for microspectrography. With these arrangements it is possible to reach the theoretical limit within the wave-length range of 30,000–2300 A., and measurements can be made in every individual wave-length. This is not the place to enter into more technical details.

When one has to study a variety of different cytological materials it is necessary to be able to study differently fixed materials. Here sources of error arise, caused by the fact that as almost all fixing agents precipitate the proteins irreversibly we get a loss of light due to light scattering in the surfaces of the protein coagulates. In the visible region of the spectrum this effect is not very important—in the ultra-violet region, however, it will spoil every effort of quantitative work if it is not properly watched for. The theoretical treatment of the optical conditions in white collodial solutions which I mentioned earlier can, however, be applied here also. This is not the place to enter into the technical details. I will, however, underline the fact that the most important of all conditioning factors for quantitative microspectrography is the elimination of the sources of errors caused by these factors. *They can be treated theoretically, and the calculations show that it is only possible to get the correct absorption curves with certain apertures after measurements in several wave-lengths close to each*



Text-fig. 1. Outline of construction of apparatus for taking absorption measurements in ultra-violet light of cell parts in microscopic sections. *A*, water-cooled high-pressure mercury lamp; *B*, tungsten lamp; *C*, monochromator; *D*, exit slit; *E*, auxiliary lens; *F*, total-reflecting prism; *G*, semi-transparent plate; *H*, condenser; *I*, object; *K*, objective; *L*, ocular system; *M*, movable prism; *N*, rotating sector adjustable with great precision while rotating; *O*, centering device; *R*, photocell; *S*, *T* and *U*, main coupling for string electrometer; *V*, auxiliary photocell which, with the aid of *G*, serves to correct variations in the source of light; *P*, rotating spark gap. The measurement procedure is, broadly, as follows. The object *I* is illuminated with monochromatic ultra-violet light from *A* or *P*. Through the quartz system *K*, *L*, *M*, the picture is thrown on *R*, whereupon the electrometer makes a deflexion. The object is then removed by means of a special apparatus, after which the amount of light falling on *R* is reduced by means of *N* until the electrometer gives the same reading as before. The loss of light in *N* is then the same as in the object. The procedure is repeated in any desired number of wave-lengths.

other (even the available mercury lines are not close enough) *and after treatment of the object in special ways and testing the optical properties of the object.* On the other hand, knowing these factors it is possible to estimate absorption curves on a variety of different objects and to make corrections for different factors and also to determine the accuracy in the individual case.

Summarizing the conditions for absorption measurements in tissues we have arrived at the following conclusion:

A quantitative determination throughout the optical spectrum of selectively absorbing substances is possible down to particles of size about four times the wave-length of the light. For doing this it is, however, absolutely necessary to control the different optical conditions. The measurements must be made with great accuracy, and necessarily in a large number of wave-lengths.

The most important of the optical conditions are the following:

(*a*) The optical system must fulfil Abbe's sine condition and furthermore be built so that all light leaving the object is transferred to the image.

(*b*) The optical properties of the object must be known in detail. Tests on the validity of Lambert Beer's law have to be made on test substances.

Any measurement in which these conditions are not fulfilled introduces sources of errors which cannot be eliminated.

The arrangements we use fulfil these conditions and have been used on different problems. The regular series of absorption studies is as a rule along the following lines:

(1) Study of the living cell in the ultra-violet microscope.

(2) Comparison of that picture with the picture of the frozen dried preparation.

(3) Measurements on the frozen dried preparation.

(4) Observation and measurements of the same preparation after extractions in different ways to correct for influences of other absorbing substances than the ones in the centre of interest, for instance, for nucleic acid and proteins, lipids of different kinds, mononucleotides, etc.

Two groups of substances are far more easily studied than almost any other group of substances, and these are the proteins, especially those containing cyclic amino-acids and the polynucleotides (see Caspersson, 1936, 1940*a*, *b*). The selective absorptions of these substances are of such an order of magnitude that, in the thicknesses of layer we have in usual histological preparations and in the concentrations in which they occur in the cells, the resulting light absorptions are for certain parts of the ultra-violet—the region between 2400 and 3000 A.—of an order of magnitude very easy to measure. Moreover, experience has shown that their absorption within this region is so high compared with most other constituents of the cell that the absorption of the others in most cases can simply be neglected at the analysis of the curves. I must underline that it is of course always necessary in every individual case to have complete absorption spectra run on differently fixed material. It is, however, a commonplace finding when starting to work on different biological materials that the other absorptions compared with nucleotide and protein absorption can be neglected. All data I have submitted below are, of course, founded on complete spectrometric studies.

The absorption spectra for the nucleic acids of different types are very similar, the selective absorption at 2600 A. being caused by the pyrimidine groups and being very little influenced by external factors such as *p*H. The detailed course of the absorption curves of the different constituents of the proteins have been studied especially in England. The most important phenomena for microspectrography are the selective absorptions of tyrosin and tryptophane. In macrospectrography Holiday has in his pioneer work used the spectrograph for amino-acid determinations by analysis of the absorption spectra. In microspectroscopy we use the procedure adopted by him. The one difference is that we often have nucleic acids present, which, however, makes no difference in principle. One detail should be mentioned especially. When the accuracy in the microspectrographic measurements was driven to 1% in many places in different cells there appeared a small but distinct displacement of the protein band in relation to the nucleic acid band. We found that the cell parts showing the displacement always were distinctly acidophilic. As the same displacement was also found in six different histone preparations, it seemed probable that it was caused in some way by the diamino-acids present, and in the early observations the substances giving their type of absorption were described as 'proteins with the absorption type of the histones'. A tentative explanation was given in 1941 (see Caspersson, 1940*b*). In order to get a better idea of the phenomenon this band displacement was studied in the chick embryo, where direct comparisons could be made with Schenk's earlier chemical data (see Caspersson, 1940*b*; Landström-Hydén *et al.* 1941; Thorell & Caspersson, 1941). He has found in the chick embryo in early stages large amounts of diamino-acids, apparently linked both in proteins chemically of histone type according to Kossel's definition and in other types of protein. In good agreement with Schenk's data the displacement was found to be more pronounced in the earlier stages during embryogenesis and less pronounced at later stages. In order to state the chemical nature in more general terms, the publications after 1941 used the name 'proteins of the absorption type of hexonbaserich proteins' instead of the earlier 'absorption type of the histones'. As the shift in the absorption band is too small to permit quantitative observations, a method was developed for the determination of the relative amounts of diamino-acids, which will be described later. A large amount of material is now available on the different objects in which at the same time the diamino-acid determination has been made as well as the absorption measurement. There is always a good correlation between these phenomena, and the shift in the absorption band can be used as a qualitative indication of the presence of high amounts of hexone bases. It should be pointed out that the physical explanation may be more complicated than the one tentatively given in 1941, as Mirsky found no shift in histone preparations completely free of nucleic acids. In the histone preparation we have as yet studied there was a very small amount of nucleic acids present, and that is the case in all biological material studied. This might mean that the presence of nucleic acids together with the diamino-acids is a conditioning factor for the shift. The nucleic acid alone causes no shift in proteins poor in diamino-acids. Whatever the mechanism might be, the shift is a very useful indication of the presence of diamino-acids in high amounts. Of course the physical nature of this mechanism does not in the least influence the validity of the microspectrographic observations on the localization of proteins and nucleic acids in general. At present all materials showing the shift mentioned in our publication have been tested with the diamino-acid localization procedure mentioned later, and in every case unusually large amounts of diamino-acid have been found.

The other procedure for diamino-acid localization is founded on the observation of Chapman, Greenberg, Schmitt and some other American investigators, who found that at a very acid reaction certain dyes reacted with the free amino groups of protein in stoichiometric relations and could be used for determination of the free groups (see Hydén, 1943*a*, *b*). We found that this staining reaction could be carried out on tissue slides and evaluated photometrically by measurements in the absorption maximum of the dye. This procedure has always been used in the studies mentioned later. Probably here a rich field for cytochemical research is waiting. As yet it is not worth while investigating further until more macrochemical data have accumulated. Dr Mirsky's most important and elegant studies will, I hope, make this field accessible also for cytochemical research.

For the nucleic acid localization it was desirable to have a suitable phosphate method, and such a procedure was worked out by Norberg (1942). With this procedure amounts as small as 10^{-7} mg. can be determined, that is, about 10^{4-5} times less than the usual so-called micromethods, and so the procedure is within the range of cytochemistry. Also determinations of different fractions can be made on objects down to single large cells or parts of very large cells, as, for instance, individual chromosomes of *Chironomus*. This procedure might easily be developed further for determinations of other substances. As, however, during the last years a new method has been developed, allowing the determination of a still increasing number of elements without combustion of the tissues (see Engström, 1946) so the work on the colorimetric procedures has been temporarily abandoned. For phosphorus the new procedure is already considerably more sensitive and accurate than the colorimetric procedure.

Finally, there should also be mentioned the Feulgen reaction which has been used to discriminate between ribodesose acids and ribose acids (see Caspersson, 1932*a*, 1936, 1940*b*, 1941).

Summarizing we can thus say that studying nucleic acid and protein metabolism we can determine the amount of polynucleotides, and cyclic amino-acids very accurately, the sum of other amino-acids with less accuracy and also get an as yet rough estimate of the relative distribution of the diamino-acids and distinguish between elements carrying nucleotides of the ribose and ribodesose type.

The study of the relations of the nucleic acid metabolism to the protein metabolism has been the main work in our Institute during the war, and I will try to present some of the general lines of the results without presenting the experimental evidence because of the limited space. I will only give a few examples of the more important tissues.

In 1939 cytochemical study with these methods of gene reproduction began. In collaboration with Dr Jack Schultz, at that time in Stockholm, we made a series of observations on *Drosophila* material which, correlated with data from other sources, led to the idea, expressed in 1939 (see Schultz & Caspersson, 1938, 1939*b*; Caspersson, 1939*a*, 1941), that the reproduction of the gene and all other self-producing protein groups could only occur in the presence of polynucleotides. The continued work has confirmed that view, and furthermore, it was shown by these cytochemical procedures, that at every opportunity, when we have the right to assume a rapid protein synthesis going on in a cell, nucleic acids are present. During the last seven years different materials have been studied, and the evidence seems conclusive that the following principles are of general validity (see especially Caspersson, 1941; see also Brandt, 1941; Brandt & Caspersson, 1941; Engström & von Hámos, 1944; Hydén, 1943*a*, *b*; Hydén & Caspersson, 1945; Hydén & Hamberger, 1945; Landström *et al.* 1941; Landström-Hydén *et al.* 1941; Malmgren *et al.* 1945; Norberg, 1942; Santesson & Caspersson, 1942; Schultz *et al.* 1940; Schultz & Caspersson, 1940; Thorell, 1944, 1945; Thorell *et al.* 1945;

Thorell & Caspersson, 1941; Thorell & Wilton, 1945; Thorell & Wising, 1944):

(1) All protein synthesis needs the presence of nucleic acids.

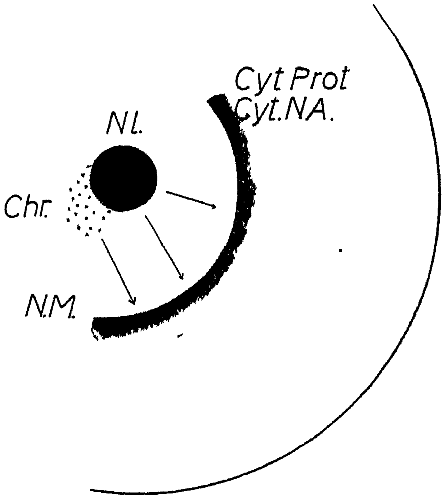
(2) Quantitatively the most important nucleic acids in the chromosome are of ribodesose nature.

(3) The nucleus itself is a cell organelle organized especially for being the main centre of the cell for the formation of proteins.

I will now indicate some of the pieces of work behind these theses. It has been performed by a group of persons, the core of which consists of Hydén and Thorell as cytologists, Engström and Norberg as chemists, Santesson and Moberger as pathologists, Malmgren and Hydén as bacteriologists, and myself. As a rule we have been working together in different combinations according to the requirements of the individual problems.

With reference to the cellular protein synthesis, it is most convenient to distinguish between two processes. The first involves the reproduction of gene proteins, which are regarded as being of many different kinds and occur within the chromosomes. The second process is concerned with the production of the main bulk of the cytoplasmic protein, which seem to be of comparatively few different kinds. The work of Spiegelman during the last few years seems to provide us with a mechanism by which the cytoplasmic proteins can secondarily get changed in different directions.

Starting to work on the cytoplasm of metazoan cells ten years ago we were at first quite astonished to find in the animal cells such enormous amounts of ribose nucleic acids, at that time in the chemical literature generally described as phytonucleic acid as opposed to the so-called animal nucleic acid—the ribodesose type. Since then data have been accumulating with other procedures, both macrochemical and microchemical. Among the latter, those of Brachet should be specially mentioned. All results show that the ribose nucleotides belong to the regular cell constituents. The ribose nucleotides were found, in fact, to be one of the most common of all cell constituents, and in 1939–40 evidence accumulated from *different lines of studies that they were connected with the synthesis of the cytoplasmic proteins.* As the result of several years of group work it was shown generally on every investigated type of cytoplasmic protein synthesis that the following mechanism worked (Text-fig. 2). A certain part of the chromatin, we call it the nucleolus-associated chromatin, secretes substances of a protein nature. There are very strong indications that they contain considerable amounts of diamino-acids. These substances accumulate and form the main bulk of the large nucleolus. From the nucleolus they diffuse towards the nuclear membrane, on the outside of which an intensive production of ribose nucleotides takes place. At the same time the amount of cytoplasmic proteins increases. Their synthesis is apparently in some way linked with the nucleic acid changes. The mechanism might look fairly hypothetical. That is, however, not the case. Different series of investigations, carried through by several workers, showed the special character of the nucleolus-associated chromatin, the composition of the nucleolus and the very close relationship between the chromatin in question and the nucleolar substances. Furthermore, the gradient in concentration of protein from the nucleolus towards the nuclear membrane has been established. There is also other evidence for protein transport of a more complicated kind. The increase in ribosenucleic acids correlated with protein synthesis in the cytoplasm is always easily shown in every case of protein increase, whether it be growth, secretion, etc. (see Brandt & Caspersson, 1941; Caspersson, 1941; Hydén, 1943*a*, *b*; Hydén & Caspersson, 1945; Hydén & Hamberger, 1945; Landström *et al.* 1941; Landström-Hydén *et al.* 1941; Malmgren *et al.* 1945; Santesson & Caspersson, 1942; Schultz *et al.* 1940; Schultz & Caspersson, 1940; Thorell, 1944, 1945; Thorell *et al.* 1945; Thorell & Caspersson, 1941; Thorell & Wising, 1944). I will now give a few examples.

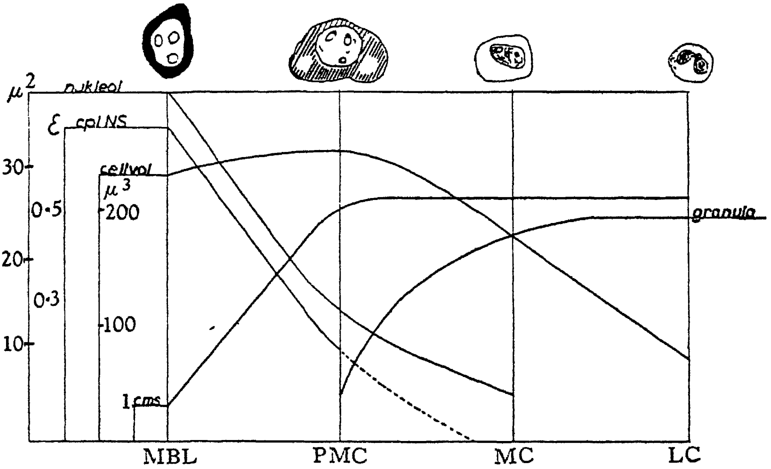


Text-fig. 2. Diagrammatic view of the cytoplasmic protein-forming system. *Chr*, nucleolus-associated chromatin; *Nl*, nucleolus. The arrows indicate the migration of proteins from the heterochromatin, direct or via the nucleolus, to the nuclear membrane, *NM*. In the vicinity of the latter the formation of cytoplasmic nucleic acid, *NA*, and cytoplasmic protein proceeds.

Let me first say quite generally that the study of a lot of different tissues, with special regard to conditions where protein synthesis processes could be assumed to occur at a higher rate than in the ordinary adult cell, always showed changes in the cytochemical organization corresponding to the outline in the diagram. It is of course not, or very rarely, possible to measure, for example, the gradient from nucleolus to nuclear membrane in the very small mammalian cell. In the picture of a cell in intensive protein production however, two phenomena are so evident that they cannot be missed, however small the cell might be, and that is the changes in the nucleolus and the appearance of the large amounts of ribose nucleotides in the cytoplasm.

The most complete series of comparisons in mammals between related cells with different rates of protein synthesis during growth is given by the blood cells, analysed by Dr Thorell (see Thorell, 1944, 1945; Thorell *et al.* 1945; Thorell & Wising, 1944). This kind of material has also the advantage that the living cells are easily obtainable and can be analysed directly. Parallel with the cytochemical studies he measured the cell volumes and the cell numbers in order to get an approximate value for the changes in the total amounts of cytoplasmic proteins during haemopoesis. By comparing the red and white blood cell series it is evident that the development of cytoplasm is far more distinct in the latter series. The cytoplasm in the red series hardly increases at all after the stage of the erythroblasts. The very first stages in both series are fairly alike and show the image of intensive function of the nucleolar apparatus according to Text-fig. 2. In somewhat later stages distinct differences occur. In the red series cytoplasmic nucleotides and nucleoli rapidly disappear, while the same are well developed even late in the white series. A rapid increase of the total mass of cytoplasmic protein also occurs at that time. Thorell has summarized his observations on a diagram (Text-fig. 3).

These data show that the changes in nucleolar mass and composition and the cytoplasmic nucleotides agree with what was expected from the diagram previously given and that is thus also valid here. Many other mammalian tissues were studied and the conditions were found to be the same. It is a fact that the different stages of blood-cell formation are so easily accessible which makes them such an excellent example. It is clear that the stage of protein formation, that is in this case, of growth, can be judged in any individual cell from the development of the corresponding organelles. Let us for the sake of simplicity call them the system for protein formation in the cytoplasm. Thorell used this when studying several different questions in haematology, normal and pathological. I will give an example. In myeloma very large amounts of plasma cells appear in the blood. They show the typical picture of intense protein production (see Thorell *et al.* 1945; Thorell & Wising, 1944). It is not possible, at present, to judge whether the protein produced is used entirely for the formation of new plasma cells or if it also passes into the blood stream, the protein level of which is very high in myeloma. A series of observations indicate that an active synthesis of this protein occurs within the cell cytoplasm. Thorell also made a study of the lymphocytes and made the observations that, especially at the stage of the large lymphocyte, an enormous synthesis of proteins occurs within the cell, the fate of which was not possible to determine cytochemically (see Thorell, 1945). Similar lines of research on protein formation have been carried out in Sweden on different questions, by different investigators, as, for instance, on different pathological processes (see Hydén & Hamberger, 1945; Santesson & Caspersson, 1942; Thorell, 1944, 1945), mode of action of vitamin C and D on regenerating tissues, etc. (see Thorell, 1945).



Text-fig. 3. Survey of some essential cytochemical and cytological data during the differentiation of a myeloblast into a mature granulocyte. *Cpl NS* = concentration of cytoplasmic nucleotides expressed in the coefficient of extinction. *Nukleol* = total nucleolar mass expressed in μ^2. *Cellvol* = cell volume in μ^3; *cms* = total volume of the different stages; *Granula* = the approximate granula content in each type of cell. The outline drawing shows how, before and during the growth phase, the cytoplasm of the stem cell (*MBL*) is packed with nucleotides, and how rapidly the concentration decreases in the course of differentiation, concurrently with a reduction of the nucleolar mass. In the later, relatively well-differentiated stages, where the nucleolus-cytoplasmic nucleotide mechanism is suppressed, the increase in the number of cells takes place at the expense of their size. The drawn cells are mainly intended to show the nucleus-plasma ratio and the nucleolus picture at different stages. In the primitive type of cell (*MBL*) the formation of a substantial nucleolar mass has broken up the nucleolus-associated chromatin into a thin outer layer. Owing to the diminished formation of nucleolar substance by the nucleolus-associated chromatin during differentiation, the outer layer is gradually converted into a compact chromocentre.

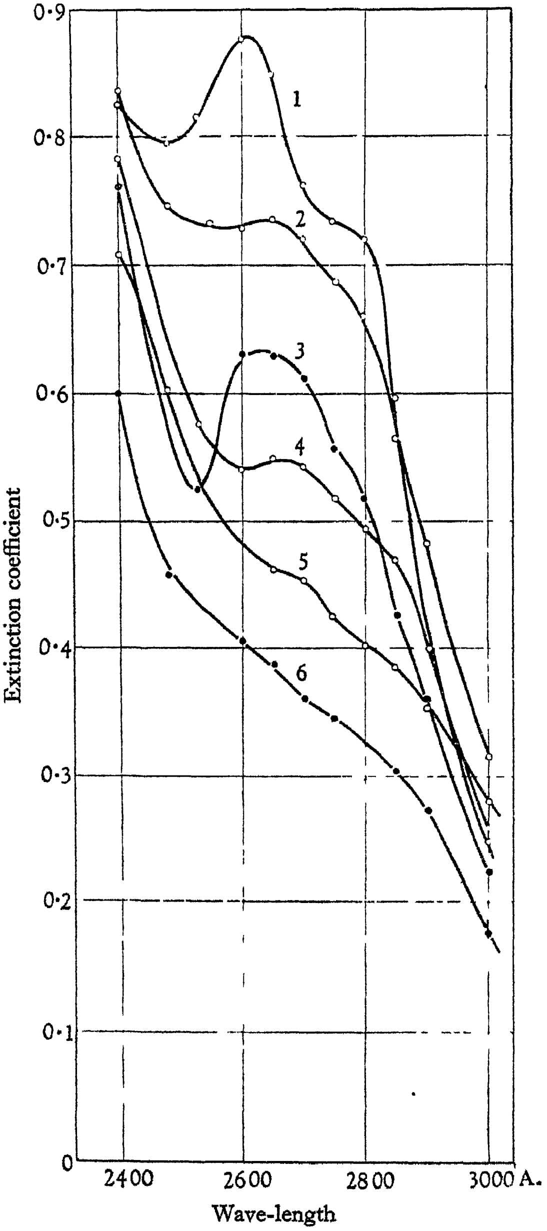
It is important to observe that the cytoplasmic protein-forming system is used not only during growth but also for production of proteins for other purposes such as secretion.

Most impressive is the protein production of different protein glands, e.g. the pancreas. The ultra-violet photograph at 2600 A. indicates the presence of heavily absorbing substances (see Caspersson, 1939*b*; Landström-Hydén *et al.* 1941). Text-fig. 4 shows as an example a set of absorption spectra of points at different parts of one single cell. Here the protein-forming system is acting intensely. A good comparison is given by the cells of the islands of Langerhans (Pl. 2, fig. 1). These cells produce protein too of course; the rate of synthesis is, however, of a much lower order of magnitude. Another striking example is given by the gastric mucosa (Pl. 2, fig. 2), where the protein-producing chief cells and the hydrochloric acid-producing cells are intermingled.

The sum of our experience, which I have tried to demonstrate with some examples, shows that for the production of cytoplasmic proteins the outline given previously is valid, and that as yet no exception has been found in spite of intense search. In comparing with other endocellular chemical processes we realize how enormously comprehensive chemically the processes must be which lead to perhaps a doubling of the amount of the principal constituent of the whole cell, the proteins, in a few hours. Then it is quite understandable that very large changes in the cytological as well as the cytochemical picture of the cell must occur.

In the adult organism there are two types of tissues which distinctly differ from the general organization of the ordinary adult cell in that they always show the picture of a cell in most intensive production. These are the egg cell and the nerve cell. The nucleolus is simply enormous, and the cytochemical structure of the cell corresponds to the composition which is to be expected from a cell in intensive protein production. For the egg cell it is quite clear that it should be so, as the increase in protein during the second period of growth is enormous.

To begin with, the nerve cell seemed very confusing. The cytochemical analyses showed that the Nissl bodies contained large amounts of ribose nucleotides, and the cell in general showed the exact picture of a cell in intense protein formation. There are, however, in the literature as yet no data on protein changes in individual nerve cells. As it seemed to be an exception from the rule it was carefully studied mainly by Hydén (see Hydén, 1943*a*, *b*; Hydén & Hamberger, 1945; Landström *et al.* 1941). He studied at first the embryonic development. The cell was shown to follow closely the general course of embryonic cells with regard to the development of the system for protein formation. In the adult stage, when the system for protein formation in other cells are breaking down, the nerve cell still keeps its appearance of a cell ready for active protein formation.



Text-fig. 4. Absorption spectra from different parts of single cells from pancreas. Measured areas each below $\frac{1}{4}\mu^2$. Curve 1 close to the cell base; curves 2, 4 and 5 more apical in the same cell.

Hydén then studied a series in which animals were forced to perform extensive muscular work. He also tried intense sensory stimulation. In both cases during function there appeared great changes in the protein content of the corresponding nerve-cell cytoplasm. The amount of protein in the individual cell could be brought down to less than one-third. That means that the major part of the cellular protein disappeared during intense functioning of the cell, and was later restored by the very same protein-forming system described previously. In the sensory stimulation the analysis showed that at the very first time there were signs of stimulation of the protein-forming system to intense activity. During the course of the very intense stimulation this was, however, overwhelmed by the demand and the protein content decreased rapidly. Experiments have been done with graded auditory stimulation where the function of the nervous apparatus can be followed at the same time by electrophysiological procedures in order to investigate the order of magnitude of these changes within the physiological range of stimulation. The present evidence leads to the conclusion that these changes in the protein metabolism in the nerve cell are normal processes correlated with nerve function.

These results were, of course, a source of satisfaction for us, as they removed the one apparent exception from the general applicability of our views. The peculiar organization of the protein-forming system in the nerve cell is thus simply explained by the fact that, correlated with its function are very intense changes in the state of its proteins, and that during intense function its protein-forming system must be able to replace used proteins very rapidly. The nerve cell is always ready to produce protein with great speed when needed. Hydén has used this observation also for the study of different normal and pathological processes in the nerve cell, such as retrograde degeneration (see Hydén, 1943*b*). It can also be used for localization experiments, etc.

All the data given above refer to the normal cell, which represents our central line of research. In order to see whether cells in pathological growth followed the same system some experiments were also tried on cancer and sarcoma cells (see Santesson & Caspersson, 1942). At first it was evident that in a cancer cell in intensive growth the system for protein formation is intensively active, exactly as was to be expected. Closer study of tumours showed, however, certain typical characteristics, namely: in the tumour cell the normal inhibitory mechanism for the protein-forming system in the cytoplasm is checked or entirely absent, and as a result the cells are recklessly 'trying' to grow as rapidly as the supply of raw materials allows. This never occurs in a normal cell where in all cases studied the cells break down their systems for protein production when the supply falls below a certain level. Thus it is possible in many cases to identify an individual cell as malignant by cytochemical analysis. The cause of the change is, of course, a quite different question. Our observation only indicates that it is to be found in the nucleolus-associated chromatin or possibly in the centres which regulate this apparatus. The first idea seems to be more probable.

In the first part of this paper I mentioned that we had to consider two processes of protein synthesis, the gene reproduction and the production of the main protein masses of the cell body (see Caspersson, 1941). As yet I have been writing mainly about the latter process. As cytochemical correlations between nucleic acid and gene reproduction were found and in a preliminary form published before the war as a result of the studies with Dr Jack Schultz, I will not enter into details but repeat some more important points (see Caspersson, 1939*a*, *b*; Schultz *et al.* 1940; Schultz & Caspersson, 1938, 1939*a*, *b*, 1940). The observations indicated that the nucleic acids take part in the process of gene reproduction. During certain disturbances of the gene reproduction, disturbances of the nucleic acid metabolism in the corresponding loci of the chromosomes were observed. Furthermore, it was found that in the *Drosophila* salivary gland nucleus the heterochromatin acted as a regulating centre for the nucleic acid metabolism of the chromosomes.

In the above-mentioned studies of the cytoplasmic protein synthesis a certain part of the chromatin was found to take part in the processes leading to the synthesis of proteins and nucleic acids. It was called the nucleolus-associated chromatin, and in the limited number of cases where it is possible to compare this chromatin with the genetically defined heterochromatin the conceptions seem to coincide. The name nucleolus-associated chromatin is chosen to avoid the name heterochromatin, as the cytological and genetical definition of the latter in ordinary nuclei still seems to be very difficult and to some extent a matter of choice. The heterochromatin seems furthermore to be a fairly complex conception.

These studies led to a preliminary model of the protein metabolism during mitosis, published in 1941 (for a general survey see Caspersson, 1939*b*), which, however, seems to lie outside the scope of this lecture. The same is the case with the different changes in the nucleic acid content of the different parts of the chromosomes connected with the cell cycle. As very little quantitative work has been done on that, it is my sincere hope that these problems will be attacked soon with quantitative cytochemical procedures.

The question of the relation between the heterochromatin and the nucleolus-associated chromatin is in my view a most important field for further cytochemical and genetical work. The present cytochemical and cytological data make it probable that, compared with the euchromatin, the heterochromatin is less complex and contains larger amounts of identical substances, call it genes. Its connexion with the main nucleolus are very close. It seems to produce the main bulk of the nucleolar substance in connexion with the synthesis of the cytoplasmic protein. It should be specially pointed out that this does not mean that all the nucleolar material is connected with this process. It might be that the synthesis of the cytoplasmic proteins is quantitatively such an extensive process that the intermediary stages, such as the formation of material appearing as nucleoli, is so prominent, that it seems to dominate the cytochemical picture. Experiments are in progress searching for similar phenomena during other processes connected with the very intense action of certain genes.

Before leaving the cell division one thing should be mentioned within parentheses. It is a remarkable fact, given from the studies with the Feulgen technique by numbers of authors, that the chromosome structure seems always to contain the specialized type of polynucleotides with very long chains, the ribodesose nucleotides. At present we can suggest two reasons for that. One might be that molecules of this shape are better 'adapted' to the polypeptide chains in the gene string, which might also mean something during the process of reproduction. The other is that assuming a very close but not exact matching in the spacings between the polypeptide chains and the nucleotide chains such as Astbury & Bell found, there is a mechanism available for the tendency for spiralization of the gene string. The experimental technique for testing this is available for many questions, in the useful procedures for studying the ultra-violet dichroism of all constituents (see Caspersson, 1940*c*; Lange, 1944, 1945).

Of more general importance for the question of the relations between nucleic acid and protein metabolism than the mitosis is the following question: the relations between nucleotide and protein synthesis seem to be evident in the metazoan cell; if the underlying principles are of general validity it is to be expected that the same mechanism should be working also in the lower organisms. The crucial objects will in this case be found within the groups of bacteria and the viruses. That question is in fact a central one for the whole idea of the polynucleotides being necessary for all protein synthesis. Because of that we have during the last few years been concentrating our work on these two groups of organisms (see Hydén & Caspersson, 1945; Malmgren *et al.* 1945).

In the study of the bacteria the very first question was as to whether there are indications of a connexion between the nucleic acid metabolism and the protein formation, and the second is the organization of that

PLATE 2

to face p. 145

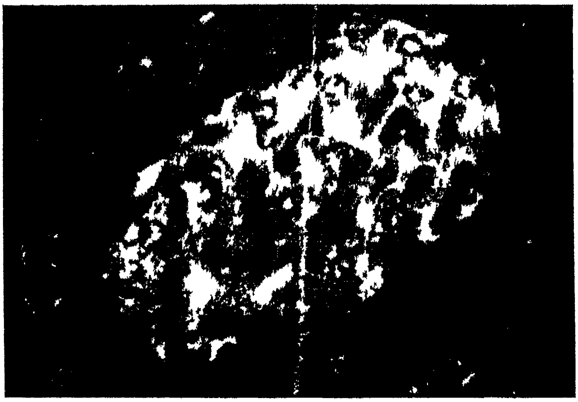


Fig 1

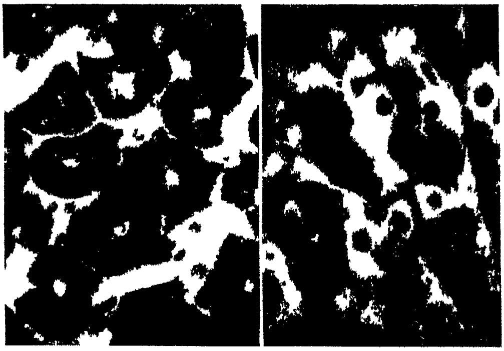


Fig 2

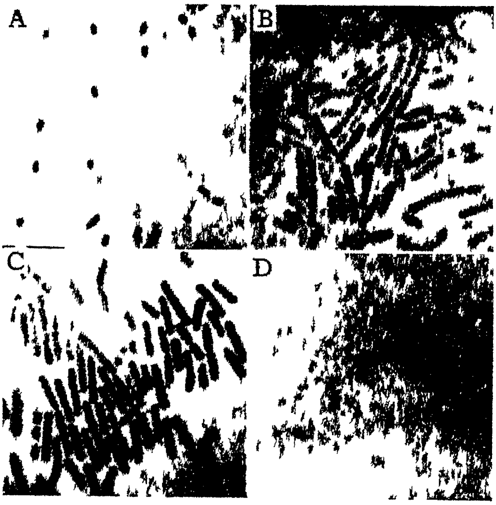


Fig. 3.

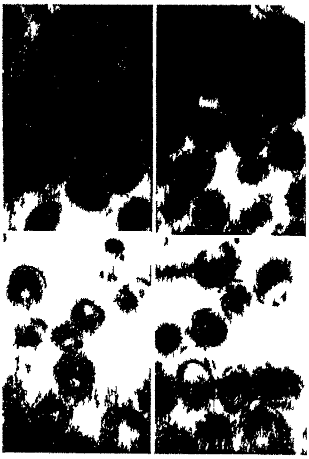
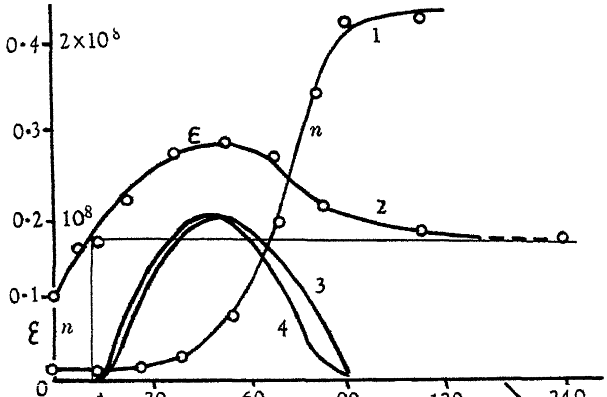


Fig 4.

system in the bacterial cell, or as it can also be expressed, the organization of the bacterial nucleus. I will not enter into the literature on the bacterial nucleus but concentrate on our experiments. The first experiments (see Malmgren *et al.* 1945) showed that very great differences in the nucleic acid content of single bacteria occur during the course of growth; furthermore, that it was possible to find conditions during which the growth could be stabilized. In order to search for a correlation between bacterial growth and nucleic acid metabolism it was for different reasons necessary to try to make determinations of the amounts of nucleic acid within the single bacterium, which proved technically to be one of the most difficult tasks we have undertaken as yet. The way found was in principle the following simple one. Absorption spectra obtained on single bacteria, correlated with extraction data, showed that within a comparatively narrow limit of error the extinction at 2600 A. could be used as a measure of the nucleic acid content of a single bacterium, and furthermore that the most important sources of error were in such a direction that they were to a large extent eliminated if we made comparisons on the same bacteria under different stages of growth. Thus series of growing bacteria were used. At different times were determined number, total nitrogen, number of living cells, length and width and the extinction on about 200 cells at every point. The results are exemplified by the following figures.

Pl. 2, fig. 3 shows a *subtilis* strain during different phases of growth. The living bacteria are photographed in the culture medium at 2570 A. A, resting bacteria; B, end of lag phase; C, intensive growth; and D, resting bacteria in the grown culture (compare growth curve 1 in Text-fig. 5). The enormous difference in light absorption at this wave-length is evident in the photographs. The quantitative evaluation of the changes gives results as presented in Text-fig. 5. Curve 1 gives the number of bacteria, curve 2 the extinction at 2570 A. of individual bacteria (each point corresponds to the mean value of 200 bacteria), curve 3 is *dn/dtn* (see below), and curve 4 the extinction curve 2 after subtraction of threshold value and multiplication with an arbitrary factor (see below).

Text-fig. 5, curve 2, shows that the first stage, the lag phase before the actual division has started, involves, as indicated by the curve, a formation of nucleotides in the bacterium. When the nucleotide content has reached a certain level, the nucleotide curve falls, and after it has reached approximately the same threshold value as when the growth started, the division ceases. If this threshold value is subtracted from the extinction curve we obtain a curve 3 showing the increase of nucleotides above the level where the growth started. By subtraction the major part of the errors due to light diffraction, etc., will be eliminated, whence the subtraction curve fairly well represents the actual changes in the content of nucleotides. The rate of growth can be computed from the growth curve as its derivate with respect to the time, dn/dt, where n is the number of bacteria and t the time. The rate at which the individual bacterium divides is thus obtained as dn/dtn, which, in this case, where the number of bacteria runs parallel with the increase in bacterial protein, is also equivalent to the rate at which the protein increase proceeds in each bacterium. If this function is constructed, multiplied by any arbitrary factor (curve 4), it will be found, within the limits of error, to correspond well with the above-mentioned nucleotide curve.



Text-fig. 5. See text for details.

This correspondence shows that *the rate of the protein synthesis in the bacteria is proportional to the nucleotide content, when the latter has reached such a value that the bacterial division has started.* The uniformity of the results in regard to the different kinds of bacteria examined indicates that this proportionality between the nucleotide concentration and the rate of protein formation is of general validity for bacteria.

The cytological analysis was made with ultra-violet microscopy and the Feulgen nucleal reaction. In all the bacteria examined a small constant organelle containing ribodesose nucleotides were observed, the behaviour of which during growth and division indicated that it corresponded to the euchromatin of higher cells.

The very first question was, as I previously said, as to whether in the bacteria a nucleic acid cycle was linked to the protein synthesis as in the metazoan cell. These data seem to show this definitely. The other question is the organization of the nucleus or its homologue. Certainly such a nucleus is expected to exist if the analogy with the metazoan cell should be correct. The easiest way to present that question is to switch over to the yeast cell (see Brandt, 1941; Brandt & Caspersson, 1941). Because of the cytological organization of the cell proper, determinations of nucleotides cannot be done as for bacteria; the absorption curves show, however, how in the growing cell the amount of nucleic acids is at a much higher level than in the resting cell. Some chemical data support that too. For yeast many observations of previous authors have shown the presence of small ribodesose nucleotide-containing chromosomes. We can support that in every case. It is striking, however, that in the ultra-violet picture of the resting cells a number of granules occur at the divisions distributed at random in the daughter cells. These granules are very rich in ribose nucleic acids. Experiments with growth at low temperatures showed that the ribose nucleotides in the cell body first appear around these granules, much in the same way as they do around the nuclear membrane in the metazoan cell. These granules thus seem in their function to correspond to the nucleolus-associated chromatin, the heterochromatin in the higher cells and centra for the synthesis of the ribosenucleic acids which co-operate in the synthesis of the proteins of the cell body. There is, however, one large difference, they do not contain any ribodesose nucleic acid, the nucleic acid specific for the chromosome. They do not, however, enter into the chromosome. Thus the organization of the yeast nucleus seems to be quite analogous to the one in the metazoan cell: it contains a gene-carrying chromosome characterized by ribodesose nucleic acid which was called the euchromatin equivalent, and, furthermore, another equivalent to the heterochromatin, or if you prefer to call it that way to the nucleolus-associated chromatin. Thus there are two structures which together correspond to the metazoan nucleus. The latter only appears as a higher developmental stage of the yeast nuclear arrangement: a stage in which a primary cell organelle for cytoplasmic protein production, the heterochromatin equivalent, has been taken up by the chromosome in order to ensure the exact redistribution of the genes for cytoplasm formation in the daughter cells.

The conditions in the bacteria investigated seem to follow that line closely. Several other authors have during the last years found a ribodesose nucleic acid containing structure in different bacteria, and we have done so too in every case investigated, which of course have to be interpreted as simple chromosomes. Granules corresponding to those in yeast are also found in the ultra-violet microscope. The bacteria are, however, so small that it is generally not possible to take absorption spectra thereof. The material is consistent with the idea that the organization of the bacterial nucleus is the same as demonstrated in yeast and thus form a phylogenetically simpler form of the metazoan nucleus, founded on the very same principles I started with, the principle of the polynucleotides being necessary for every protein synthesis and being of two kinds, of which one, the ribodesose nucleic acid, is a specialization of the general mechanism, adapted to suit the chromosome.

Thus the bacteria seem to fall into the same general lines as the metazoan cell. The next question is: What is the case in the still simpler organism as, for instance, viruses of different orders of organization? We have here to make a difference between the very simplest ones, consisting, as we know, of only one type of protein, as the simplest plant viruses and the poliomyelitis virus, and the higher ones which seem to form a transition to the group of the bacteria. The simpler types of virus have according to the chemical analyses ribonucleic acids present. A series of higher viruses has been studied cytochemically (see Hydén & Caspersson, 1945).

It appeared that all the viruses interacted with the host cell in the way that they were parasites on the system for protein production. When we analysed this system in an individual host cell it could be shown that different viruses intrude upon different phases in this system. It was then possible to study the virus elements in the cells at the time of their growth, and in the more complicated viruses we investigated it was evident that at their reproduction a ribodesose nucleotide mechanism was working, that is, the general nucleic acid mechanism for protein synthesis and the specialization thereof for the reproduction of several proteins linked together, the chromosome mechanism. The simplest viruses consisting of, as far as we know, just one single type of protein contains also nucleic acid but of the ribose type.

How is the organization of the virus to be explained along the general lines mentioned earlier?

The observations on the nucleic acid metabolism in the infected cell show that all viruses studied appear as parasites on the system for protein formation of the host cell, indicating that their own system for that is incomplete. The larger viruses contain, during the period where you can see they reproduce, ribodesose nucleotides; the very simplest ones contain ribosenucleotides. The explanation seems simple. The chromosome mechanism is the way Nature has found to distribute genes of a number of different kinds evenly among the daughter individuals, and the essential thing is that the different groups are linked after each other in long chains, the gene string. The nucleic acid mechanism is working at different kinds of protein synthesis, but on the gene string there appears a special kind of polynucleotide, the ribodesose type. The higher viruses are more complicated than the simplest ones and must be assumed to contain several different proteins or genes, and it is to be expected that an arrangement exists ensuring their proper distribution to the daughter individuals—that is, the chain in a simple chromosome. In correspondence with that, we find the specific chromosome nucleic acid—the ribodesose acid—on the higher viruses. In the simplest virus, consisting of one single kind of protein, there is, of course, no need for such a mechanism, and thus the simplest general mechanism works, the ribose nucleotide mechanism.

Thus it seems possible to follow the general principles of nucleic acid being necessary for the biological protein synthesis and of the ribodesose polynucleotide being a specialization for the gene string throughout the whole series, from the simplest organisms to the highest, and that a large part of the well-known cytological phenomena in different cell nuclei merely reflect the fact that its chief task is to be the main, of course not only, centre in the cell for the synthesis of the cell proteins of different kinds. These principles also give an explanation of the organization of the cell nuclei in different stages of the phylogenetic development.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Absorption spectra of 10μ thick layers of 2% nucleic acids (2% N.S.) and of 10% protein, serum globulin (10% Äggv.). The lowest curve gives a general idea of the average absorption of other substances in an ordinary mammalian epithelium or connective tissue cell.

Fig. 2. *Tradescantia* pollen mother cell, photographed in the living state at 2570 A. The very heavy absorptions are caused by the ribodesose nucleotide content of the chromosomes.

Fig. 3. Root cells from *Allium*. The dark cells from the growth zone near the tip, the light cells from the part which is not growing. The heavy absorption in the cytoplasms of the former cells are caused by ribose nucleotides present in high concentrations.

PLATE 2

Fig. 1. Photograph at 2570 A. of a Langerhans island from mouse pancreas.

Fig. 2. Gastric mucosa. 2570 A.

Fig. 3. Photographs identically treated of the same culture of *B. subtilis* at different times. A, at the beginning of the experiment; B, at the end of the lag phase; C, period of intensive growth; D, when growth has ceased.

Fig. 4. Upper left: yeast cells in living state, commercial baker's yeast. Upper right: the same culture after starvation. Lower right: the same strain in intensive growth. Lower left: the same strain fermenting but not growing (nitrogen-deficient medium). 2570 A.

PROTEIN AND NUCLEOTIDE METABOLISM IN THE NERVE CELL UNDER DIFFERENT FUNCTIONAL CONDITIONS

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In the following paper some examples are given to illustrate changes in the content of protein and nucleotides in nerve cells in connexion with motor, sensory and psychic function. These examples, which are briefly reported, are chosen from a series of cytochemical investigations on the nervous system carried out at the Karolinska Institutet, in Stockholm, during 1940–6. For all details and technical data the reader is referred to the original papers.

I. PROTEIN INCREASE IN NEUROBLASTS AND YOUNG NERVE CELLS DURING DEVELOPMENT

The nerve cells occupy a unique position among the cellular structures in the organism. Mitosis in the nervous system ceases at an early stage of development. During the following stage the neuroblast's growth is considerable to produce a cell with a large nucleus and cytoplasm, usually equipped with several processes. The total volume of the processes, exclusive of the myelin sheath, frequently exceeds that of the cytoplasm many times.

If one supposes that (1) in mammals an anterior horn cell, simply calculated as a prolonged rotation ellipsoid, has a mean diameter of 50μ and a nuclear diameter of 18μ, and that (2) the axon has a mean diameter of 8μ, exclusive of the myelin sheath, and a length of 8 cm.; then the volume of the axon will exceed that of the cytoplasm about 1000 times. A complete neuron is the greatest structure in the organism, both if you include the axon or if you do not. The demands of protein production during embryological development for building that structure must thus be very high.

Initially the nerve cell develops in the same manner as other somatic cells. But from the multipolar neuroblast it passes through a period of growth which can be described as enormous. In order to compute the amounts of protein and nucleic acids in the cytoplasm of the embryonal nerve cell at different stages of development cytochemical analyses were carried out on material from the spinal cord from a total of ninety-nine

PLATE 1

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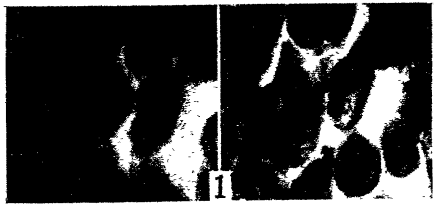
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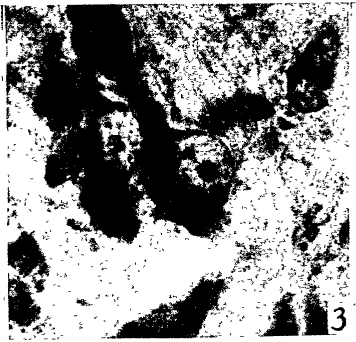
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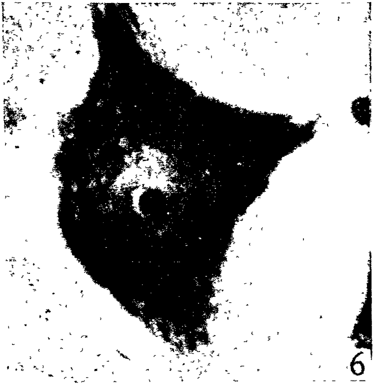
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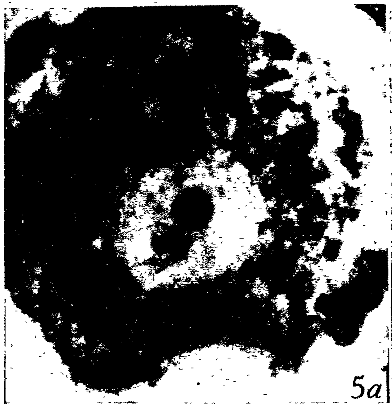
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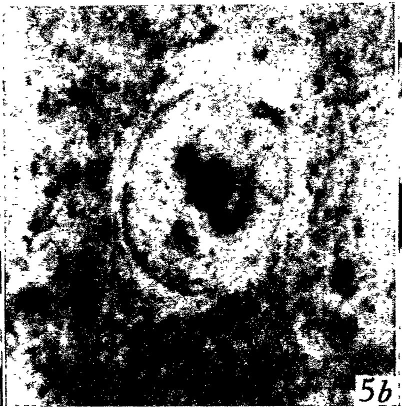
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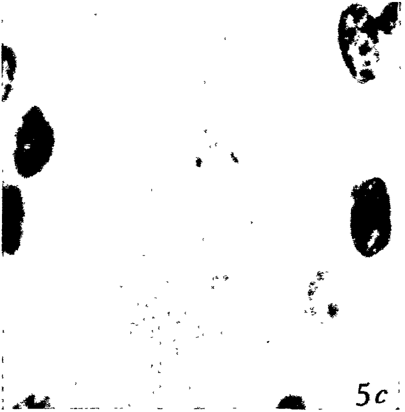
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For explanation see p. 161

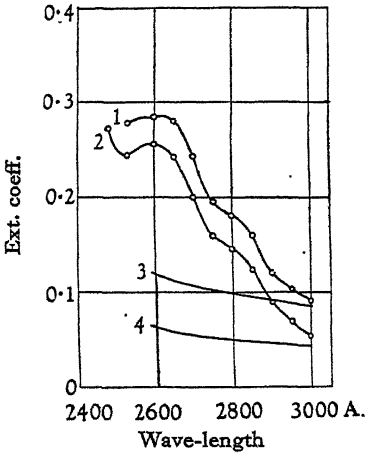
embryos, including human, rat and rabbit embryos (Hydén, 1943*b*). The quantitative determinations were based on complete absorption curves from 2400 to 3100 A. from different points in the cytoplasm of the nerve cells. For the theoretical background and technical details in the ultra-violet microspectrography, as well as other complementary cytochemical methods used in these experiments, reference should be made to the paper by Caspersson in the present volume and to the original papers (see, for example, Caspersson, 1936, 1940; Hydén, 1943*b*). For convenience in comparing the data the amounts of tyrosine and tryptophane obtained in the absorption measurements have been reckoned on the basis of a protein of standard type containing an average content of 5% tyrosine and 1·5% tryptophane. In order to show the differences in the amount of protein in the embryonal nerve cell at different stages of development, the cell volume in the various instances was computed under the assumption that the cells were prolonged rotation ellipsoids. The largest and smallest cell diameters were taken as axis values in a section of the cell containing the major part of the nucleolus and consequently passing through the centre of gravity of the cell. The section of the cell also contained cytoplasm at the base of the neurite, which afforded a guarantee that the large axis would correspond with the maximum diameter of the cell.

Pl. 1, fig. 1, shows a group of unipolar neuroblasts from a 10 mm. rat embryo, unstained and photographed near the absorption maximum of nucleic acids at 2570 A. These cells have a very scanty cytoplasm, the major part of which is arranged as a cap on the nuclear pole. The cytoplasm has a high absorption at that wave-length. Text-fig. 1 gives an example of absorption curves from points in the cytoplasm of unipolar neuroblasts. At 2600 A. a distinct absorption maximum appears, due to the pyrimidine and purine rings in the polynucleotides which could be shown to be of the ribose type. At 2800 A. another absorption maximum is visible, attributable mainly to the component amino-acids, tyrosine, tryptophane and phenylalanine. The quotients ϵ 260/ϵ 280 in eighteen absorption curves from different unipolar neuroblasts in the same section showed that the ratio of the concentration of protein to the nucleic acid ranged from 3 : 1 to 5 : 1. The protein concentration as referred to the standard protein was about 30%.

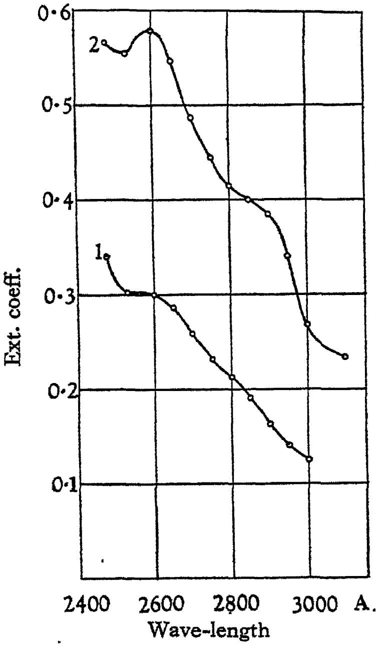
Pl. 1, fig. 3, gives an example of a young anterior horn cell from a 23 mm. rat embryo photographed at 2570 A. Text-fig. 2, curve 2, shows an absorption curve from a point in the cytoplasm of the same cell. The quotients ϵ 260/ϵ 280, computed with due allowance for the light scattering in the preparation from fourteen different anterior horn cells, signified that the ratio of the concentration of the protein to the nucleic acids varied from

8 : 1 to 11 : 1. Microspectroscopical analyses of thirty adult anterior horn cells from the anterolateral group in the spinal cord from rats were performed, and the analyses of the absorption spectra showed an average of 25–30 % protein and 2–3 % nucleic acids.

The analyses of absorption spectra from points in the cytoplasm showed thus that in the unipolar neuroblasts of rats the content of protein was about 30 % and in adult anterior horn cells from the same animal likewise about 30 %. If the increase of the cell volume from the unipolar neuroblast to the adult anterior horn cell is estimated, it appears that the total amount of proteins in the nerve-cell cytoplasm increases more than 2000 times during development. If the axon is included in the calculation the values are increased to between 30,000 to 200,000 times, depending upon the length of the axon.



Text-fig. 1.



Text-fig. 2.

Text-fig. 1. Spectra 1 and 2 from points in the scantily developed cytoplasm in unipolar neuroblasts. Curves 3 and 4 show the estimated loss of light by scattering.

Text-fig. 2. Curve 2 absorption spectrum from a point in the cytoplasm of the young anterior horn cell in Pl. 1, fig. 3.

Corresponding to these large demands in protein production the young nerve cell contains during the whole period of development a large nucleolus rich in polynucleotides together with cytoplasmic nucleotides in high concentrations. Thus the nerve cell is from the very beginning intended for intense protein production. This capacity remains even in the adult stage (Landström, Caspersson, Wohlfart, 1941; Hydén, 1943*b*), a feature which is unique among the different tissues of the mammalian organism. Further studies showed, however, that this peculiar organization is explained by the fact that during the function of the neuron large and rapid changes occur in the content of protein in the cytoplasm of the nerve cell.

II. THE COMPOSITION OF CERTAIN PARTS WITHIN THE NERVE CELL NUCLEUS

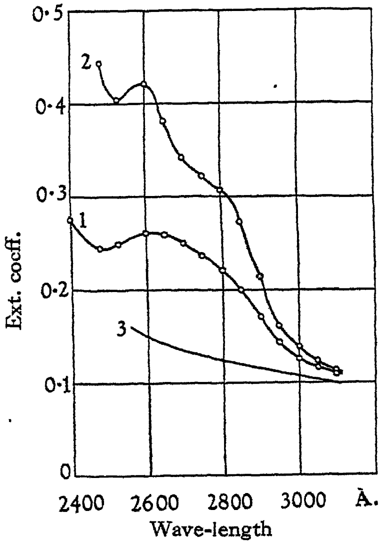
In view of the importance of the heterochromatin in protein production in cells (cf. Caspersson, 1939, 1941) and its derivative, the nucleolus-associated chromatin, and the significance of that cell organelle in the nerve cells (Hydén, 1943*a*, *b*; Hamberger & Hydén, 1945) a brief description of the organization and composition of the nucleolus-associated chromatin in the nerve cell will be given before discussing the experiments dealing with nerve cells under different functional conditions.

In the nerve cell near the nucleolus is a region with a higher absorption at 2600 A. than is present in the remainder of the nuclear substance (Pl. 1, fig. 5*a*). This region within the nucleus contained proteins rich in basic groups and small concentrations of ribose nucleotides. The protein concentration referred to the standard protein was on an average three times that of the remaining nuclear substance, exclusive of the nucleolus. These areas of high protein concentration belong to the nucleolus-associated chromatin. In Pl. 1, fig. 5*b*, is seen a spinal ganglion cell from a rabbit. The cell is treated with cyananthrol RBX, an acid dye, in the presence of a surface-active medium and photographed at the absorption maximum of that dye at 5750 A. in order to determine the amount of free amino-groups. The photograph shows the great capacity of the nucleolus and the nucleolus-associated chromatin for binding acid-dye groups, and it demonstrates the close relationship between these two organelles. The nucleolus-associated chromatin in the nerve cell is separated in two parts: one part is the above-mentioned area in the neighbourhood of the nucleolus (Pl. 1, fig. 5*a*, *b*) and the other part which immediately surrounds the nucleolus contains ribodesose nucleotides (Pl. 1, fig. 5*c*).

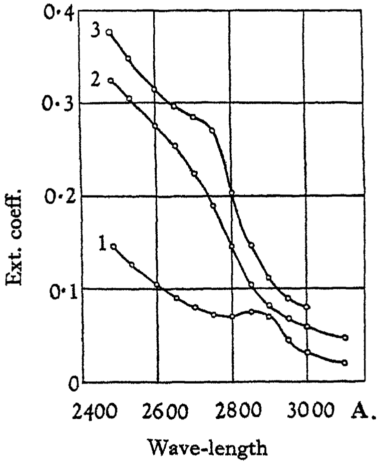
III. MOTOR STIMULATION ON ANTERIOR HORN CELLS

In the course of intense muscular work with increased function, there occurs a sharp decrease in the content of protein and nucleic acids in the cytoplasm of the anterior horn cells. The difference in the protein and nucleotide content of the nerve cells between the animals at rest and in a state of exhaustion is considerable, amounting to between three and five times (Hydén, 1943*b*).

The experiments were performed on eight guinea-pigs, four of which were used as control animals. The animals were induced to run on a revolving belt until exhausted. The lower part of the cervical and lumbar intumescence were taken for examination. For comparing the data obtained from exhausted animals and animals at rest, the root cells in the nucleus anterolateralis of the myorhabdotic group, which were found to have a constant composition, were used. Altogether forty-six series of measurements were carried out. Sixteen complete spectral analyses were made, and in the other instances ϵ 260/ϵ 280 was computed with due allowance for the light scattering and by referring to the standard protein. The latter type of determination can only be used as a crude complementary method to the complete absorption measurement between 2400 and 3100 A. Pl. 1, fig. 6, shows a nerve cell, photographed at 2570 A., and belonging to the nucleus anterolateralis from a guinea-pig at rest. The whole cell absorbs intensely, and curve 2 in Text-fig. 3, which is taken from a point in the cytoplasm, shows a well-defined band at 2600 A. and another at 2800 A. Pl. 2, fig. 8, shows a corresponding cell from a guinea-pig in exhaustion. The cell absorbs less, and curves 2 and 3 in Text-fig. 4 are taken from points in the cytoplasm of this cell. The absorption maximum at 2600 A. has disappeared. The analyses of the spectra obtained showed that the content of protein and



Text-fig. 3.



Text-fig. 4.

Text-fig. 3. Absorption spectra from points in the nucleus (curve 1) and cytoplasm (curve 2) in the anterior horn cell in Pl. 1, fig. 6, from a guinea-pig at rest.

Text-fig. 4. Absorption spectra from points in the nucleus (curve 1) and cytoplasm (curve 2) in the anterior horn cell in Pl. 2, fig. 8, from an exhausted guinea-pig.

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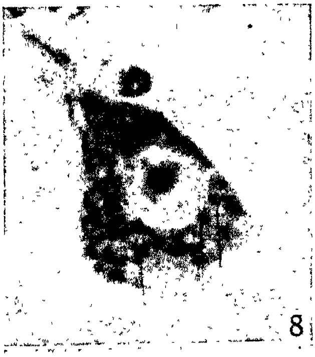
PLATE 2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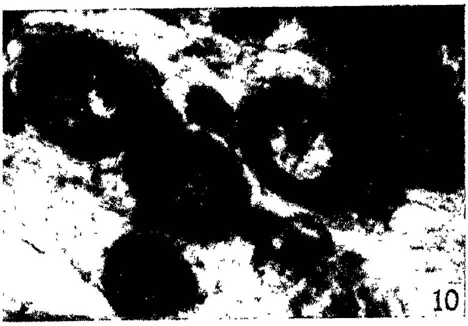
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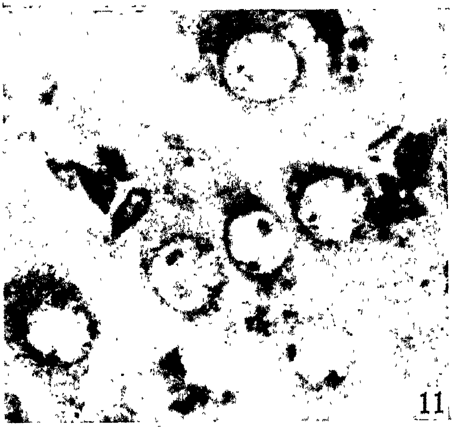
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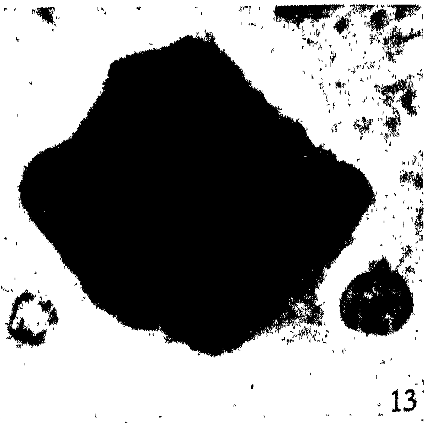
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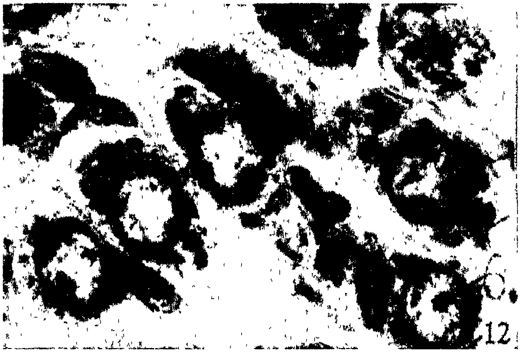
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nucleotides in spinal motor nerve cells from guinea-pigs at rest were three to five times greater than from animals in exhaustion.

These experiments show that during intense muscular work, with increased function of the respective myorhabdotic cells, diminution in the content of protein and nucleotides occurs. The original values were restored during the first 48 hr. after exercise. This signifies that the function of the nerve cell is accompanied by metabolic processes involving extensive quantitative changes in the protein and nucleotide content.

In this connexion I will emphasize that if nerve cells are stained with basic dyes of different kinds according to the ordinary staining methods the intensity of the colour will not necessarily correspond to the content of polynucleotides. It is easy to alter the intensity of the staining of a nerve cell with basic dyes by slight changes in the technical procedures employed. A nerve cell rich in nucleotides, for example, can be very faintly stained or a cell poor in nucleotides can be stained intensely in that way. One has to be very cautious about drawing conclusions regarding the content of nucleotides in a nerve cell from their staining intensity. Quantitative data can of course only be calculated from a complete absorption curve taken between 2400 and 3100 A.

IV. SENSORY STIMULATION ON NERVE CELLS

As an example will be mentioned an experiment performed on 146 guinea-pigs employing acoustic stimulation and subsequent investigation of the cytochemical changes in the nerve cells of the cochlear ganglion (Hamberger & Hydén, 1945). One advantage with these nerve cells is their uniformity in the cytochemical composition under acoustically quiet conditions. The cytoplasms of the nerve cells throughout the ganglion have a nucleic acid concentration of about 2 % and a protein concentration of between 20 and 30 % as determined by reference to the above-mentioned standard protein. In order to study the magnitude of the changes within the physiological range of stimulation the more obvious changes in the function of the ear have been followed by means of electro-audiograms.

On acoustic stimulation with tones of various frequencies and intensities the ganglion cells undergo extensive cytochemical changes. On exposure, for example, to a tone of a frequency of 6000 cyc./sec. and intensity of 80 db. for 3 hr. the ganglion cells pass through a cycle of changes within 3 weeks. Pl. 2, fig. 10, taken at 2570 A., shows a group of nerve cells from the cochlear ganglion of guinea-pigs under acoustically quiet conditions. The spectral analyses showed an average concentration of 2–3 % nucleic acids and 25–30 % protein. Following the stimulation, the cellular protein and nucleotides diminishes, the diminution being most marked during the second week. Pl. 2, fig. 11, is a photograph of a group of nerve cells from the cochlear ganglion taken 2 weeks after the stimulation with the above-mentioned tone for 3 hr. Spectral analyses showed that most of the nucleotides had disappeared and the protein concentration decreased from 25–30 to 2–8 %. This difference, with due allowance for the slight difference in size between non-stimulated and stimulated cells, was statistically valid. As compared with non-stimulated animals the electro-audiogram recordings showed no decrease in the readings. In the third week the original protein and nucleotide concentration is restored. The first sign of this phase is an accumulation in the cytoplasm about the nuclear membrane of ribose nucleotides and proteins with a marked basic character (Pl. 2, fig. 12). The electro-audiograms recorded showed no decrease in the values as compared with those for non-stimulated animals.

Acoustic trauma caused by reports from a revolver likewise entails diminution in the cellular protein content. In that case, however, there are signs of damage to the cytochemical organization of the cells. This diminution is not completely restored even after the lapse of 8 weeks after the experiment, although most of the cells by that time show signs of restoration. The electro-audiograms recorded concurrently showed a marked decrease which indicates a disturbance in the auditory function caused by the acoustic trauma.

The results of the experiments with acoustic stimulation also lead to the conclusion that the changes in the protein metabolism in the nerve cell are normal processes correlated with function.

V. CHANGES IN THE CONTENT OF PROTEIN AND NUCLEOTIDES IN THE NERVE CELL AFTER EXCISION OF THE AXON

In addition to Bodian's paper (cf. Gersh & Bodian, 1943 and this number of this journal) on nerve-cell regeneration, I refer to the experiments on the same subject which have been carried out in Stockholm (Landström-Hydén, 1942; Hydén, 1943*b*; Hydén & Rexed, 1943). After excision of the axon considerable changes were found in the content of protein and nucleic acids in the cytoplasm of both motor and sensory nerve cells. During the next few days after the excision of the axon, the content of nucleotides and proteins is reduced, and after about a fortnight the cytoplasm of the cells has lost most of the nucleotides and proteins. After about a month the content of protein in the cells begins to be restored. The first signs of this restoration are the appearance of protein of a markedly basic character and of ribose nucleotides in the cytoplasm about the nuclear membrane. This phenomenon has been established in every case where protein production in nerve cells is increasing from a low level, and can be regarded as a sign of intense protein production in nerve cells (cf. Hydén, 1943*a*).

Gersh & Bodian (1943) claim that they have not been able to show any decrease in protein in the cytoplasms of nerve cells after excision of the axon. They have based their calculation of nucleotides on the assumption that no protein is lost by the cytoplasm during chromatolysis.

This divergence in the findings of Gersh & Bodian and our data can be explained by the deficiencies in their technique regarding the quantitative determinations of nucleic acids and protein. They base their calculations only on measurements of the extinction coefficients at 2804 and 2650 A., without due allowance for the sum total of light losses in the section. The great importance of performing complete spectral analyses in quantitative determinations of nucleic acids and protein in single cells and the significance of the quoted conditions in analysing the spectra obtained has been strongly emphasized (cf. Caspersson, 1936, 1940; Hydén, 1943*b*, 1944; Hamberger & Hydén, 1945).

VI. THE CONNEXION BETWEEN PROTEIN METABOLISM IN THE NERVE CELLS AND PSYCHIC FUNCTION

From both theoretical and practical points of view it should be of interest to investigate whether it is possible to influence the content of nucleotides and proteins in nerve cells by chemical substances. A suitable substance was found in malonitrile, $CH_2(CN)_2$. It was found in experiments started in 1944 that after administering this substance to animals considerable changes occurred in the cytochemical composition of the nerve cells in the central nervous system. The experiments with malonitrile, which have also been carried out in man, will be published in detail elsewhere (Hydén & Reuterskiöld, 1946). The intention in mentioning these experiments here is not to advertise a new therapeutic method in mental diseases, only to report a biological experiment with importance in the discussion of protein metabolism and function.

The toxicology of malonitrile has long been known and the detoxicating effect of sodium thiosulphate on animals poisoned by malonitrile was carefully studied by Heymans & Masoin (1897).

If malonitrile is administered to animals in sufficient doses, about 4 mg./kg. body weight, a great increase in the content of protein and nucleic acids in nerve cells can be observed. This effect is already noticeable in both cytoplasm and nucleus 1 hr. after intravenous treatment. The amount of nucleotides in the cytoplasm of motor cells was doubled after treatment. This difference was statistically significant. The protein content of the cytoplasm was almost doubled, and the newly produced protein contained a high amount of basic groups. These changes in the cytoplasmic protein were also statistically valid. Pl. 2, fig. 13, photographed at 2570 A., gives an example of a motor cell from a rabbit treated with malonitrile. It was not possible to show this effect on cells belonging to other tissues than the nervous system.

These experiments gave an opportunity to show on a quite different biological material, namely, on human beings, the connexion between protein metabolism and function in the nerve cells.

The reported experiments on animals showed that protein and nucleotide metabolism in the nerve cells were associated with their function. We therefore thought it possible that a stimulation of protein production in the nerve cells likewise would entail a stimulation in function in those cases where the protein production was low. In cases of manic-depressives and schizophrenia of long duration it was found that the content of protein and nucleotides in various kinds of nerve cells was low. The material was collected both from healthy and sick people, partly in connexion with leucotomy operations and partly post-mortem within 3 hr. after death.

After treatment with malonitrile, in doses from 3 to 5 mg./kg. body weight, most cases—we have tried it in twenty-nine cases with up to ten treatments in each case—showed varying degrees of beneficial psychic stimulation. In most of the psychotics, who had been ill up to 20 years, some of the catatonic signs disappeared and they became more mentally stimulated. This effect was noticeable within 48 hr. after treatment and remained up to 10 days. Most remarkable were three cases of acute depression where the depressive symptoms ceased within 1 hr. after the starting of the treatment. These were the only cases of acute depressions which had hitherto been treated.

With this brief report I only wish to stress the main point in this experiment: that the correlation between protein metabolism in the nerve cells and function which was shown in animal experiments also seems to be confirmed regarding the psychic function.

VII. SUMMARY

There are reported some cytochemical experiments on nerve cells during development and under different functional conditions. The experiments show that metabolic processes involving extensive quantitative changes in the content of protein and nucleotides of the nerve cells are going on in connexion with motor and sensory function. This correlation between protein metabolism in the nerve cells and function seems also to be observed in relation to the psychic function.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. A group of unipolar neuroblasts with a cap of intensely absorbing cytoplasm. Objective aperture 0·85. Condenser aperture 0·6. Wave-length 2570 A. × 1150.

Fig. 3. Young anterior horn cell from a 23 mm. rat embryo. Objective aperture 0·85. Condenser aperture 0·6. Wave-length 2570 A. × 1150.

Fig. 5. *a*, spinal ganglion cell from rabbit. Objective aperture 0·85. Condenser aperture 0·6. Wave-length 2570 A. × 1150. *b*, spinal ganglion cell, rabbit, stained with cyananthrol RBX in the presence of a surface-active agent and photographed at 5750 A. × 1400. *c*, spinal ganglion cell, rabbit, on which the Feulgen reaction has been performed. The nucleolus is surrounded by particles containing ribodesose nucleotides. × 1200.

Fig. 6. Anterior horn cell of nucleus myorhabdoticus from a guinea-pig in relative inactivity. Objective aperture 0·85. Condenser aperture 0·6. × 1150. Wave-length 2570 A.

PLATE 2

Fig. 8. Anterior horn cell of nucleus myorhabdoticus from an exhausted guinea-pig. Objective aperture 0·85. Condenser aperture 0·6. × 1150. Wave-length 2570 A.

Fig. 10. A group of nerve cells from the cochlear ganglion of an acoustically non-stimulated guinea-pig. Objective aperture 0·85. Condenser aperture 0·6. × 1200. Wave-length 2570 A.

Fig. 11. A group of nerve cells from the cochlear ganglion of a guinea-pig killed 2 weeks after acoustic stimulation with 6000 cyc./sec. and 80 db. for 3 hr. Objective aperture 0·85. Condenser aperture 0·6. × 1200. Wave-length 2570 A.

Fig. 12. A group of nerve cells from the cochlear ganglion of guinea-pigs killed at the beginning of the third week after acoustic stimulation with 6000 cyc./sec. and 80 db. for 3 hr. A distinctive feature of this stage is the appearance of intensely absorbing rings round the nuclei, the nuclear-membrane nucleotides, which are typical of this stage of incipient regeneration. Objective aperture 0·85. Condenser aperture 0·6. × 1200. Wave-length 2570 A.

Fig. 13. Spinal motor cell from a rabbit treated with malonitrile, 4 mg./kg. body weight, and killed 1 hr. after treatment. Note the intense absorption power. Objective aperture 0·85. Condenser aperture 0·6. × 1200. Wave-length 2570 A.

NUCLEIC ACID IN NERVE-CELL REGENERATION*

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I. INTRODUCTION

The adult nerve cell possesses a unique structure which recommends it for experimental studies of cellular mechanisms. Its axon, an extension of its cytoplasm and often enormously elongated, can be conveniently amputated in certain instances so as to modify sharply the structure and metabolism of the cell body. Severance of the axon process of some nerve cells in the central nervous system results in irreversible changes leading to cell dissolution in 1 or 2 weeks. Other central neurons respond by assuming a condition of persistent atrophy, without regrowth of the axon. Nerve cells whose axons lie in peripheral nerves, however, respond to amputation of a large cytoplasmic mass by profound but reversible structural and metabolic changes which may lead to complete axon regeneration. In these cells the adjustment of cellular mechanisms to a suddenly reduced cell mass, and the subsequent process of cytoplasmic regrowth, may be studied in an adult cell in which cell division does not occur. Moreover, the induced changes in levels of important chemical components and of enzymatic activity in the cell can be compared with normal levels and correlated with altered functions. The induced changes are best studied in the motoneurons of the anterior columns of the spinal cord for several reasons. First, these neurons constitute a large population of cells of relatively uniform size and structure, as compared, for example, with the heterogeneous population of sensory ganglia. Secondly, after cutting a spinal root or peripheral nerve, such as the sciatic, in which lie the axons of cell bodies on the corresponding side of the spinal cord, the altered cell bodies can be compared with normal cells on the opposite side (Text-fig. 1 A and Pls. 1 and 2). This is of great importance in histochemical studies, for the modified nerve cells and their normal controls can be studied in the same histological section after identical treatment. Moreover, a population of motoneurons, more or less uniformly modified by spinal root section on one side, can be similarly compared with the opposite control side by analysis of tissue suspensions. Following the method of Howe & Mellors (1945), the right and left anterior columns of

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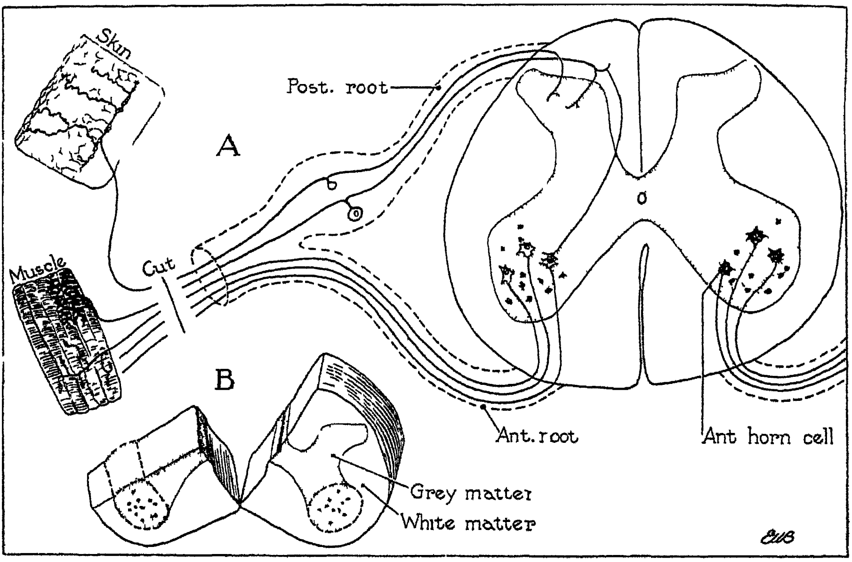
cervical or lumbar enlargements of the spinal cord can be isolated separately. This is accomplished by freezing the whole cord block on carbon-dioxide ice immediately after removal from the living anaesthetized animal, and then dissecting out the cylindrical columns in the frozen state by scraping away white matter with a sharp scalpel (Text-fig. 1 B). Each anterior column of a limb segment contains the cell bodies of about 14,000 neurons, one column normal and one modified by section of spinal root or peripheral nerve. The columns may then be prepared as suspensions for analysis, the two sides differing only in the changes in motoneurons induced by axon interruption. Neuroglial elements, blood vessels, and nerve fibres of passage remain unaltered. Such a sample in the rhesus monkey, which was used in all of the work to be described, weighs about 80 mg. Of this mass, probably no more than 4 mg., or one-twentieth of the total, consists of the motoneurons being studied.



Text-fig. 1. A. Diagram of spinal cord illustrating the relationship of cell bodies in anterior grey horns (anterior horn cells) to the long cytoplasmic extensions (axons) supplying the muscles. The axons are shown severed as they pass from the nerve root toward the periphery. Sensory axons supplying skin or muscle have cell bodies in the spinal ganglion associated with the posterior root. B. Diagram illustrating the method used to obtain anterior grey columns for chemical study of suspensions. In the frozen state the cord is split, the dorsal quadrants removed (left), and finally the white matter scraped off, leaving the cylindrical column containing the anterior horn cells (in dotted outline). The procedure is described in detail in the text.

The changes in the nerve-cell body which occur after axon amputation have been studied by histologists since 1892 when Nissl described the most prominent change, the dissolution of the basophilic masses which bear his name. The dissolution or chromatolysis of these structures, the Nissl bodies,

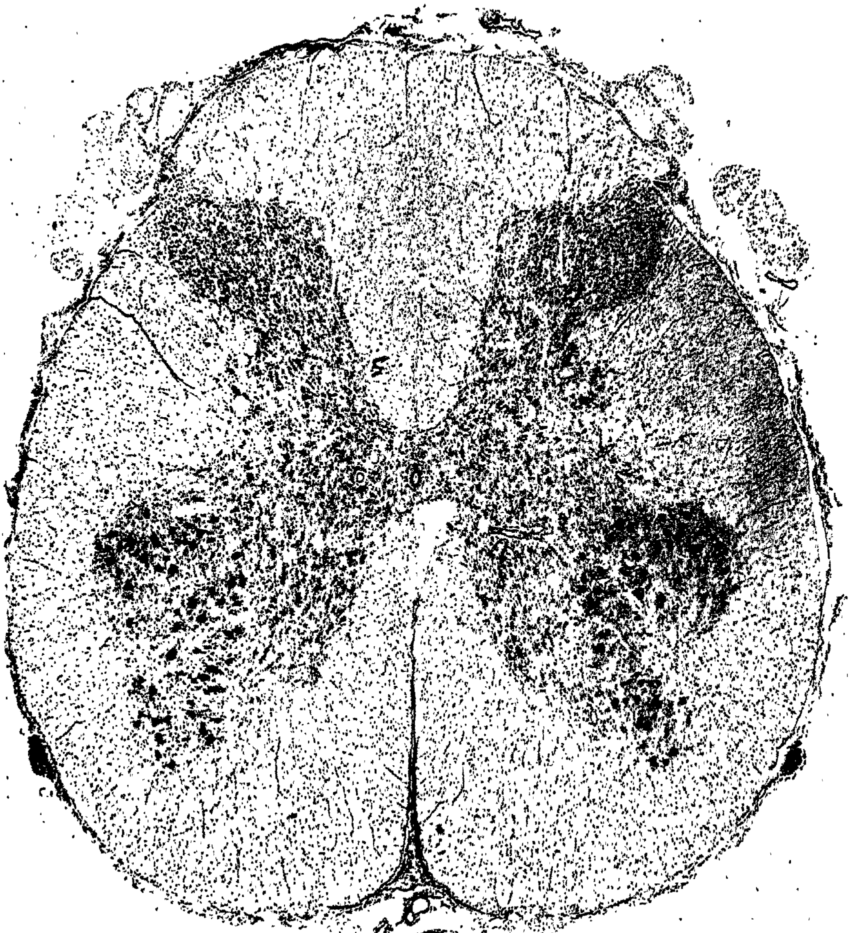


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PLATE 2

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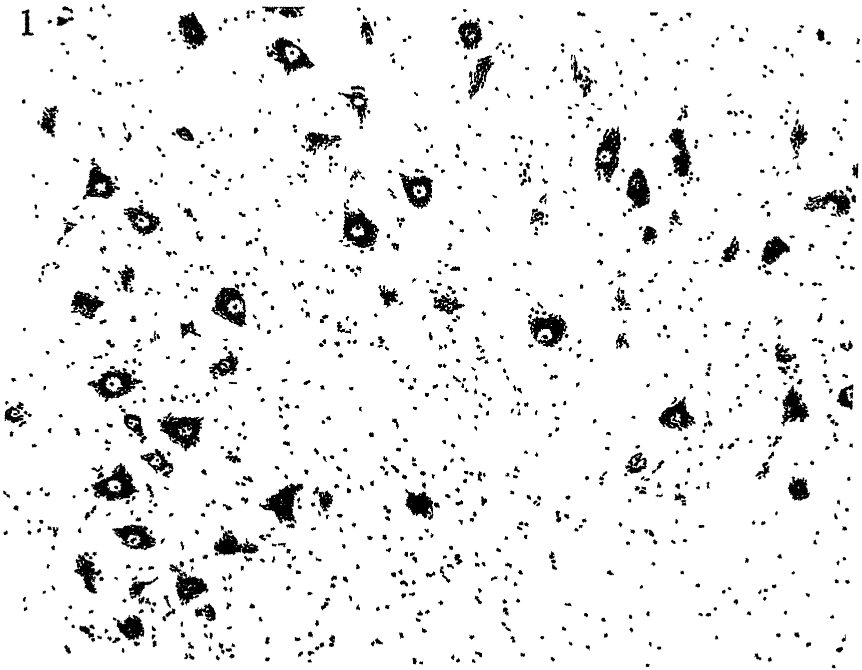

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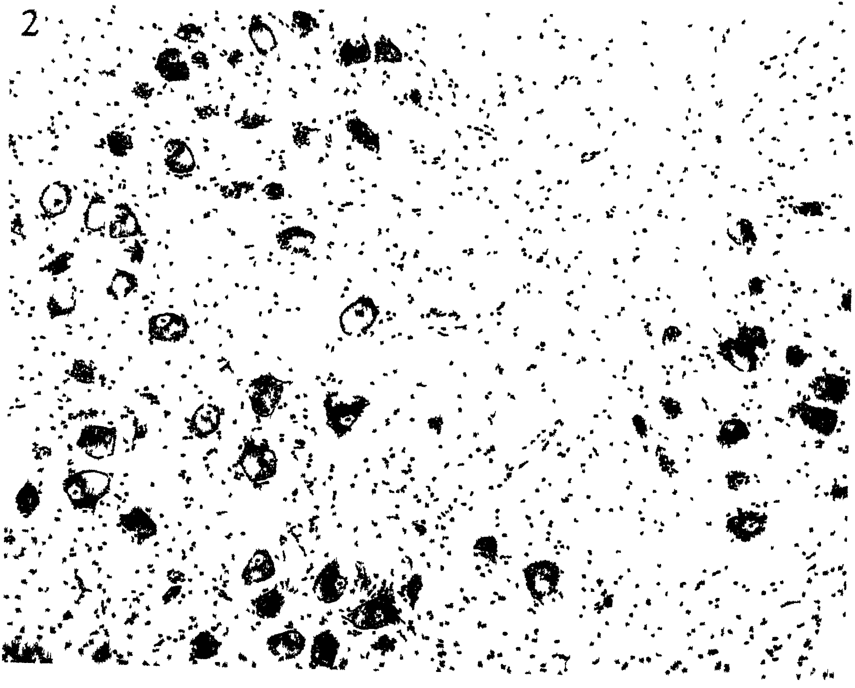

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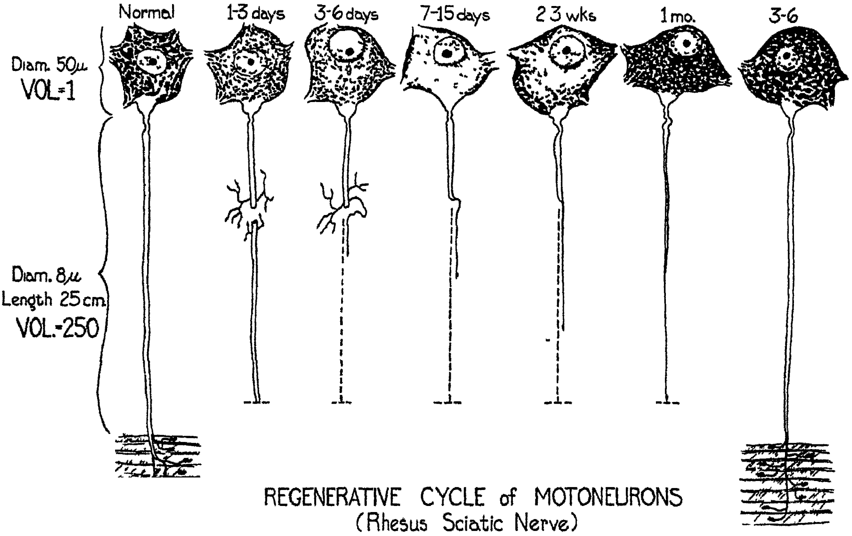
is slowly reversible, and the cycle of change is of special interest because the Nissl bodies are apparently cytoplasmic nucleoprotein structures. Although the histologist Hans Held early suggested in 1895 that the Nissl bodies consisted of nucleoprotein, on the basis of staining properties, solubility, and phosphorus content, it remained for Caspersson (1940) and his coworkers to confirm this thesis with an independent method. The high specific absorption of Nissl bodies at about 2600 A., and their negative reaction with Feulgen's 'Nucleal' staining, indicate that a high concentration of nucleic acid occurs in the Nissl bodies, and that the nucleotide components of the nucleic acids involved are of the pentose type (Landström, Caspersson & Wohlfart, 1941).

Gersh & Bodian (1943), moreover, found that the basophilic staining of Nissl bodies was lost after treatment of fixed sections with Kunitz's (1940) crystalline ribonuclease preparation, leaving the protein structure of the Nissl bodies relatively intact. This confirmed the earlier work of Brachet (1940). We also found that after the enzyme treatment the peak of absorption at 2652 A. disappeared, but a general decrease in absorption from 2400 to 3100 A. also occurred.

In summary of the contemporary evidence, it appears likely that nerve-cell bodies have an unusual concentration of cytoplasmic pentose-nucleic acid, and that this nucleic acid is identifiable histologically as the basophilic component of Nissl bodies. Nevertheless, the isolation of Nissl bodies and their direct chemical characterization are obviously urgently necessary. This leads us to consider the metabolism of nerve-cell nucleic acid, and the perplexing question of the function of cytoplasmic nucleic acid in the normal and regenerating economy of the cell.

Although our material of choice is difficult to work with in many ways, it has the advantage of permitting a study of a cycle of great but controllable changes in the growth functions of a non-dividing adult cell. I shall try to integrate the results obtained from anatomical, chemical, histochemical, and virus studies, but since our principal preoccupation has been with events at the cellular level, the approach to the chemical problems will be in the nature of sorties rather than frontal attacks.

Regressive changes in neurons follow various disturbances of the steady state, such as those induced by chemical agents, toxins, virus activity, and other agencies. Under certain conditions disturbances induced by any of these means may lead quickly to death of the cell in a very short time. The Nissl substance appears to be the most sensitive morphological indicator of regressive changes, since it regularly shows the first changes, and these are in the direction of reduction in quantity. Although it remains to be seen whether important chemical changes are altered in a parallel manner in the chromatolysis induced by different agencies, we have concentrated our attention primarily upon the regressive and recovery changes induced in nerve cells by a readily controllable agency, axon section (Text-fig. 2). This operation imposes two necessary adjustments in the over-all metabolism of the nerve cell. We know that the axon is vitally dependent upon the cell body for its maintenance, for if it is severed, the distal part, after 2 or 3 days, degenerates. This means that in a normal motoneuron, in which the volume of axoplasm may be several hundred times that of the cell body, some mechanisms in the cell body must be geared to maintain the total cell mass. When the bulk of the cell mass is amputated by axon section, it seems apparent that some adjustment to greatly decreased total cell volume must occur, and that this adjustment must be shortly followed, if not overlapped, by the requirements of axon regeneration. These relationships were clearly visualized and stated by Heidenhain in 1911. The adjustment to permanently decreased total cell mass can be illustrated by the finding of Weiss, Edds & Cavanaugh (1945) that in a capped peripheral nerve in which fibres cannot reach their end-organs, the proximal portions of such fibres undergo marked and persistent reduction in calibre.



Text-fig. 2. Diagram illustrating the cycle of changes of cytoplasmic Nissl bodies (nucleoprotein), correlated with axon amputation and regeneration. Note the disproportionately large volume of axoplasm, containing no Nissl bodies, as compared with the cell body (see photographs in Bodian & Mellors, 1945).

Moreover, some nerve cells in the central nervous system, after failure of regeneration of severed axons, atrophy and persist as small cells (Howe & Bodian, 1941). In the period of adjustment to axon section, changes have been studied by two groups who have attempted to correlate morphological with chemical events. Hydén (1943) in Caspersson's laboratory and Gersh & Bodian (1943) using Caspersson's ultra-violet absorption method, have presented evidence that the decrease of Nissl substance, so characteristic of the regressive period after axon section, is correlated with a decrease in the chromatolytic cytoplasm of absorption of light at 2650 A., as well as a smaller decrease in the protein band at 2800 A. It seems likely that the decrease at 2650 A. represents primarily a decrease of pentose nucleotides which are part of the Nissl body structure. The decrease and replacement of this cytoplasmic component can also be readily followed in ordinary histological preparations, and these changes in the cell body may be correlated roughly with the phases of axon regeneration (Text-fig. 2).

Within 24 hr. after axon section, reduction in the amount of Nissl substance is apparent, and this reduction then proceeds rapidly for a period of about a week. During this period, both the protein structure of the Nissl body, which is separately stainable with eosin, after ribonuclease treatment has removed the basophilic components (Gersh & Bodian, 1943), and the basophilic component itself, seem to melt away in the central portion of the cell body. These protein changes seem to be reflected in a decrease in absorption at 2804 A. I had assumed that the vigorous early sprouting from the central stump might be responsible for this depletion, because of demands of axoplasmic synthesis, but, although this may play a role in the regressive phenomena in the cell body, it is also possible that some of the reduction of Nissl substance represents an adjustment to the altered requirements of a decreased cell mass. In any event, the recovery changes of Nissl substance are visible within 2 weeks after axon section, when the largest part of the task of axon regrowth is yet to be accomplished. One may postulate that the recovery period involves a gradual restoration of the balance of nucleoprotein degradation and synthesis, and this recovery requires several months in contrast to the rapid course of the regression period. Nevertheless, normal quantities of Nissl substance seem to be resynthesized before axon volume is quite restored.

In summary of the morphological data, I should like to emphasize the two phases of adjustment of the 'Cytochromatin', as Heidenhain has termed the Nissl substance, to changes in cell mass. First, the regressive period, in which decrease of Nissl substance occurs, follows axon section by 1 or 2 weeks. The decrease is inversely proportional to the distance from the cell body of axon section, and is therefore proportional to the decrease of total cell volume. Secondly, the recovery period, in which increase of Nissl substance occurs, varies from 3 to 6 months, depending on the severity of chromatolysis. It is largely coincident with the period of reconstitution of most of the original cell mass. It should be recalled that during the early part of the period I have termed 'regressive', vigorous axon sprouting occurs from the central axon stumps, as well as abortive sproutings from the central end of the distal stump. Although I earlier believed that these sproutings could represent a significant phase of cytoplasmic synthesis, I now feel that their quantitative importance, in comparison with the regrowth of the bulk of the axon mass which occurs during the recovery period of Nissl substance, remains to be demonstrated.

II. CHANGES IN NUCLEIC ACID DURING CHROMATOLYSIS

Studies of absorption of ultra-violet light at 2650 and 2800 A. by chromatolytic cells and by normal controls, following Caspersson's histochemical method, have been made by Hydén (1943) and by Gersh & Bodian (1943). Since further work which was indicated at that time was prevented by the war, my summary of the high points of our work will include only data already published. The outstanding finding in our study, as in Hydén's, was a decrease of extinction coefficients at 2652 and at 2804 A. in chromatolytic cytoplasm as compared with normal. The decrease paralleled the morphological changes in the Nissl bodies, and levels returned toward normal in the recovery period. A second important finding was that the relatively constant relationship between absorption at 2652 and at 2804 A. that occurs in normal cells, and which is represented by the slope of the curve between these two points, does not hold in chromatolytic cells. On the contrary, the curve flattens after the peak of chromatolysis occurs, and this relatively greater decrease of absorption at 2652 A. persists through the early recovery period until Nissl body aggregates begin to become prominent again. The curve, however, never flattens to the degree seen after ribonuclease treatment of normal cell cytoplasm. It is important to emphasize the differences in our approach as compared with that of the Stockholm investigators. Hydén described fairly complete curves, with as many as a dozen points from 2400 to 3200 A., in relatively few cells. To compensate for normal variation, which we demonstrated in even the relatively homogeneous anterior horn-cell population, we used large groups of cells and measured only the significant peaks at 2652 and 2804 A., after assuring ourselves that the complete curves in normal cytoplasm matched those obtained for individual Nissl bodies by Caspersson (1940). Hydén studied induced changes in cells of the sensory ganglia, in which it has long been known that normal variation in distribution of Nissl bodies in the cytoplasm is great. We chose the anterior horn cells in which Nissl body distribution is as constant as can be found in any large population of nerve cells (compare Pls. 2 and 3). Hydén fixed his material by immersion in Carnoy's fluid or by the freezing-and-drying method of Gersh. Our material was fixed by intravascular perfusion of fixative in the anaesthetized animal, which eliminates many of the artefacts of immersion fixation, and also avoids the ice-crystal artefacts of freezing-drying. Finally, our measurements were made of regions of cytoplasm 6·65 μ in diameter, to reduce the variation from cell to cell due to variegated shapes of Nissl bodies. Hydén measured smaller areas.

In spite of these differences of method it is of interest that the findings in the two laboratories with respect to the principal changes in the cytoplasm are in agreement. We also found changes in the nucleoli during chromatolysis which paralleled those apparently occurring in the Nissl bodies. We did not study changes in the nuclear sap. Those mentioned by Hydén did not enable us to evaluate the extent of normal variation, and consequently of induced changes. The concentration of Nissl substance near the nuclear membrane in the recovery period is a phenomenon that is well known, and Hydén has shown a correlation with the concentration of absorbing groups. His interpretation of this phenomenon as indicating a primary role of nuclear and nuclear membrane nucleotides in determining the protein synthesis in the cytoplasm is open to the following objections in the case of the nerve cell.

First, the aggregation of Nissl substance in the recovery period near the nuclear membrane is not a constant phenomenon, and is not seen in many cells. Secondly, in all cells we have studied there is a marked aggregation of Nissl substance near the cell membrane with an increase in amount at this site during the recovery period. It might appear that either the cell membrane or the nuclear membrane may be the site of accelerated metabolic activity. I can then only repeat our conclusion previously expressed that the hypothesis of a protein synthesis process in the regenerating nerve cell occurring only in relation to the nucleolus or nucleus is as yet not confirmed.

The nucleoprotein changes in the cytoplasm of the cell body which occur during regression and regeneration, seem very likely, nevertheless, to be associated in some way with the adjustment of the steady state of the nerve cell to the initial decrease of cell volume and to the subsequent growth requirements of the regenerating axon. In this connexion one may cite the pertinent finding of Weiss (1944) that axoplasmic damming occurs proximal to a constriction, as evidence for continual growth of the axon. Such growth, or perhaps one should say replacement, is perhaps dependent upon the metabolism linked with nucleic acid turn-over in the cell body.

It is unfortunate that along with available data on rate of increase of axon length we do not have quantitative data regarding the rate of increase of axon volume during the period of axon regeneration, since this might be a useful morphological measure of the work demands of protoplasmic synthesis imposed on the cell, and could be compared with embryonic rates. We must assume, I believe, that both the rate of regrowth and the final axon volume have limits determined by the metabolism of the definitive neuron cell body, since Sanders & Young (1944) have shown that regrowing axons will not exceed their normal calibre even if larger Schwann tubes are available.

III. ENZYMATIC CHANGES DURING CHROMATOLYSIS

In order to correlate enzymatic changes with the changes of nucleoprotein levels in the cycle of chromatolysis, our laboratory has thus far carried out preliminary studies of activity of certain oxidative enzymes and of phosphatase. Using suspensions of anterior grey columns of the spinal cord, prepared as described above, Howe & Mellors (1945) showed a definite decrease in the activity of cytochrome oxidase in chromatolytic motoneurons as compared with normal motoneurons. The decrease was present in the regressive period and was even more marked in the early recovery period. These results are yet more striking when it is realized that the anterior horn-cell population in which the change occurs constitutes at most about 4 mg. of the 80 mg. of grey matter in a sample. Howe & Flexner in an unpublished study have shown a parallel decrease in succinic dehydrogenase activity.

With Flexner, I have tried to demonstrate changes of acid phosphatase activity in similar suspensions, using the method of Fiske & Subbarow (1925) for determination of liberated phosphorus. Changes of phosphatase activity, using sodium glycerophosphate as substrate, were, however, within the limits of precision of the method, a finding which was to be anticipated from knowledge of the results with the histochemical phosphatase method (Table 1). It will be recalled that Mellors and I were able to demonstrate an increase of acid phosphatase activity in the cytoplasm of chromatolytic cells, using the histochemical method devised by Gomori (1939), as modified by Wolf, Kabat & Newman (1943). The increase occurred only in the cell-body cytoplasm in the region of Nissl body changes, and was first detectable in the late stages of the regressive period. As far as could be determined, the levels declined to normal in the cytoplasm only in the final stages of recovery of Nissl bodies. The increased phosphatase activity is therefore correlated in time principally with the recovery period of Nissl bodies, or with accelerated nucleoprotein resynthesis. It is interesting that changes in phosphatase activity were not detected in the nucleus at any phase of the regenerative cycle. The substrates used were sodium glycerophosphate, adenylic acid, and nucleotides from yeast nucleic acid at *p*H 5·0. Since the increase was detectable histochemically only in the cell-body cytoplasm, which comprises about 5 % of the total volume of the anterior grey column, it is possible that the localized changes were too small for detection in suspensions of anterior grey.

There remained, however, a possibility of further analysis of the regenerating cell with the biochemical phosphatase method. The proximal part of a severed nerve contains regenerating axons imbedded in about eight times their volume of supporting tissue elements. These supporting elements undergo little or no visible changes. The axons also appear unchanged in histological sections, except for a small reduction in calibre. Since the axons contain no Nissl bodies, their separate analysis permits a study of the phosphate metabolism of nerve-cell cytoplasm free from Nissl body nucleoprotein, although smaller amounts of dispersed nucleic acid could be present. Our studies, although incomplete, have yielded results which encourage further exploitation of this approach. In the period of 10–14 days after sciatic nerve section, the proximal part of the nerve, containing regenerating axons, doubles its acid phosphatase activity as compared with normal nerve (Table 1). This increase seems attributable only to the axoplasm, since it is the only component of the proximal stump which may be expected to be affected by the nerve section. If this assumption is correct it follows that acid phosphatase activity increases in the whole regenerating nerve cell. This generalized increase was not revealed by the histochemical method, so that we may postulate that this method is only sensitive to much larger changes, or at worst is unreliable for even the

Table 1. *Acid phosphatase activity (p*H 5·0) *in suspensions of normal and regenerating nerve tissue of rhesus*

(P/mg./hr.)

Normal anterior grey	Chromatolytic anterior grey	White matter of cord	Normal sciatic	Regenerating sciatic 10–14 days	Wallerian degeneration 10–14 days
0·387	0·398	0·078	0·083	0·131	0·409
0·412	0·301	0·191	0·041	0·140	0·305
0·391	0·343	0·057	0·053	0·093	0·395
0·415	0·330	0·030	0·060	0·115	0·304
0·352	0·326	—	0·068	—	—
0·353	0·312	—	0·033	—	—
0·388	0·384	—	0·057	—	—
—	—	—	0·064	—	—
Av. 0·385	0·342	0·089	0·057	0·120	0·353

crudest quantitative estimates. Dr Flexner and I have found, for example, that every step in the preparation of histological sections for histochemical phosphatase study sharply reduces the phosphatase activity as determined with the biochemical method. Nevertheless, the apparent local increase in phosphatase activity in the chromatolytic portion of the regenerating cell may be of a higher order than that in the axon, and therefore demonstrable histochemically.

An interesting sidelight on the role of acid phosphatase in other growth processes has turned up in our study of suspensions of the distal part of a severed sciatic nerve at 10–14 days of Wallerian degeneration. Analysis of such suspensions was intended as an additional control for the study of the regenerating stump and of the normal nerve, since in the distal stump the axons are almost completely degenerated at this time. It was found that acid phosphatase activity was six times that of normal sciatic nerve (Table 1). This increase must be attributed to the proliferation of Schwann cells, which reach their maximum growth rate at this time, according to Abercrombie & Johnson (1946). These interesting relationships of phosphatase activity with growth changes in regenerating and degenerating nerve merit further investigation.

IV. CHANGES IN CREATINE PHOSPHATE DURING CHROMATOLYSIS

Pursuing the correlation in time of increased phosphatase activity and of increased nucleic acid turnover, Mellors and I initiated an analytic study of important acid-soluble phosphate compounds in regenerating and in normal anterior horn cells, using the sampling method of Howe & Mellors (1945). In order to prevent post-mortem autolysis, the cord segments were exposed by laminectomy, quickly removed from the anaesthetized animal and instantly plunged into an alcohol-dry ice mixture. The cord was placed on dry ice for removal of the anterior horns, and kept frozen until extracted with chilled trichloracetic acid. This study was interrupted by the war, but certain findings were of considerable interest. In a preliminary way, we chose to deal first with inorganic phosphate, acid-hydrolysable phosphate, shown by Kerr (1942) to be largely adenosine triphosphate, and creatine phosphate, using the method of Fiske & Subbarow (1925), as adapted for use with the photoelectric colorimeter by Stone (1943). Even with the small samples of anterior column grey matter, satisfactory checks were obtained in comparing right and left sides of normal animals, although considerable variability in levels was encountered from animal to animal. The averages of groups of animals, however, were comparable with the values obtained by Stone in dog brain with much larger samples.

The most important finding was an average decrease of phosphocreatine of about 40% in regenerating anterior grey as compared with normal grey of the opposite side (Table 2). A parallel series of normal controls showed an average sampling difference of 9% between right and left sides, and in the opposite direction. Calculation of the standard error of the mean difference in the experimental series showed that the difference was 4·5 times the standard error, and therefore well beyond the sampling error. Since no significant difference in results was obtained in cervical as compared with lumbar spinal cord, or in the 30-day as compared with 6-day regenerating cord, the results from all are combined in the calculations and in the tables.

Table 2. *Phosphocreatine in anterior horn of monkey spinal cord*

(mg./100 g. tissue)

Control series					Experimental series				
Exp.	Normal left	Normal right	Difference	% Difference	Exp.	Regenerating left	Normal right	Difference	% Difference
Cervical cord					Cervical cord				
A717	18·2	16·8	+1·4	+ 7·7	A738	5·6	7·4	− 1·8	−24·3
A719	9·7	10·2	−0·5	− 4·9	A739	4·3	8·0	− 3·7	−46·4
A754	5·8	6·2	−0·4	− 6·5	A797	12·5	10·9	+ 1·6	+14·5
A730	16·6	15·2	+1·4	+ 8·4	A778	8·9	20·6	−11·7	−56·7
A667	12·8	10·6	+2·2	+17·2	A779	4·6	16·8	−12·2	−72·0
A777	13·7	14·4	−0·7	− 4·9	A780	6·1	31·8	−25·7	−81·0
A806	14·3	14·0	+0·3	+ 2·1	A677	5·0	15·9	−10·9	−68·6
A774	22·9	14·2	+8·7	+38·0	A684	18·0	22·6	− 4·6	−20·4
A773	7·9	10·1	−2·2	−21·8	A559	9·8	17·6	− 7·8	−44·3
					A688	10·6	15·2	− 4·6	−30·2
Lumbar cord					Lumbar cord				
A717	23·2	19·0	+4·2	+18·2	A738	11·5	16·3	− 4·8	−29·4
A754	7·1	5·9	+1·2	+16·9	A739	3·6	4·8	− 1·2	−25·0
A730	29·5	26·4	+3·1	+10·5	A797	15·2	16·3	− 1·1	− 6·8
A667	21·7	20·7	+1·0	+ 4·6	A778	8·1	9·9	− 1·8	−18·2
A777	12·7	12·5	+0·2	+ 1·6	A779	6·3	14·2	− 7·9	−55·5
A806	8·4	7·8	+0·6	+ 7·2	A780	16·6	29·2	−12·6	−43·1
					A802	13·1	22·4	− 9·3	−41·4
Mean	15·0	13·6	+1·37	+ 9·1	Mean	9·4	16·5	− 7·1	−43·0
Sigma mean difference		0·64					1·53		

It was found, too late for repetition, that the values for inorganic phosphorus were too high because of unforeseen hydrolysis of acid-hydrolysable phosphate during the determination. The adenosine triphosphate values were therefore so low that our failure to find a difference between regenerating and normal sides cannot be considered significant. Similarly, no significant difference in inorganic phosphate between normal and regenerating anterior grey was found. Table 3 summarizes the differences in the combined values of inorganic phosphate and adenosine triphosphate. The decrease of creatine phosphate in regenerating neurons, when considered in the light of apparently normal levels of inorganic phosphate, suggests an increased turn-over of creatine phosphate.

Table 3. *Sum of inorganic and acid-hydrolysable phosphate in anterior horn of monkey spinal cord*

(mg./100 g. tissue)

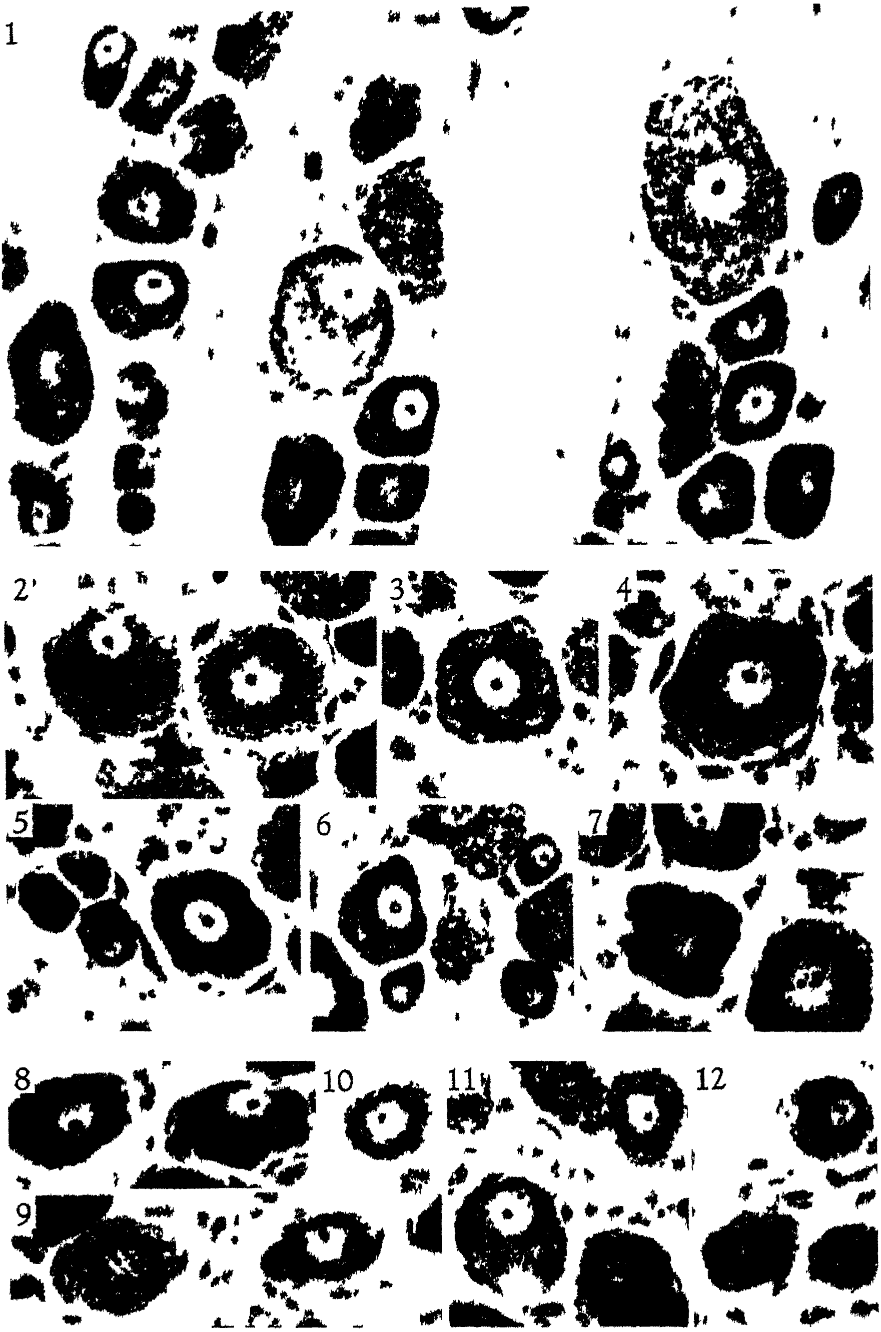
Control series					Experimental series				
Exp.	Normal left	Normal right	Difference	% Difference	Exp.	Regenerating left	Normal right	Difference	% Difference
Cervical cord					Cervical cord				
A717	24·9	22·1	+2·8	+11·2	A738	20·8	23·2	−2·4	−10·3
A719	28·4	24·8	+3·6	+12·7	A739	23·8	22·9	+0·9	+ 3·8
A754	23·5	24·6	−1·1	− 4·5	A797	20·6	20·0	+0·6	+ 2·9
A730	15·2	21·4	−6·2	−25·3	A778	25·2	23·7	+1·5	+ 6·0
A667	21·7	20·4	+1·3	+ 6·0	A779	20·2	23·7	−3·5	−14·8
A777	20·2	19·0	+1·2	+ 5·9	A780	19·6	20·8	−1·2	− 5·8
A806	21·1	21·8	−0·7	− 3·2	A677	23·6	25·6	−2·0	− 7·8
A774	22·0	24·7	−2·7	−10·9	A684	21·0	21·2	−0·2	− 0·9
A773	18·4	20·6	−2·2	−10·7	A559	31·6	31·0	+0·6	+ 1·9
					A688	25·2	24·0	+1·2	+ 4·8
Lumbar cord					Lumbar cord				
A717	23·2	21·9	+1·3	+ 5·6	A738	23·7	24·7	−1·0	− 4·0
A754	25·0	26·0	−1·0	− 3·8	A739	23·1	27·0	−3·9	−14·5
A730	17·8	18·7	−0·9	− 4·8	A797	22·9	23·3	−0·4	− 1·7
A667	21·6	21·1	+0·5	+ 2·3	A778	22·0	27·9	−5·9	−21·1
A777	21·9	21·8	+0·1	+ 0·5	A779	21·9	19·2	+2·7	+12·3
A806	25·6	22·8	+2·8	+10·9	A780	22·7	21·9	+0·8	+ 3·5
					A802	25·1	27·1	−2·0	− 7·4
Mean	22·0	22·1	0·1	− 0·5	Mean	23·1	24·0	0·9	− 3·8

V. CHANGES IN RESISTANCE TO VIRUS ACTIVITY IN CHROMATOLYTIC CELLS

The finding by Howe & Bodian (1941) that regenerating nerve cells exhibit an increased resistance to the destructive action of poliomyelitis virus, was the original incentive for our studies of nerve regeneration. It was found that this increased resistance occurred most strikingly during the recovery period. In highly susceptible cells like the anterior horn cells, axon section at least 2 weeks before infection protected the chromatolytic cells, whereas all other anterior horn cells were destroyed by virus activity.

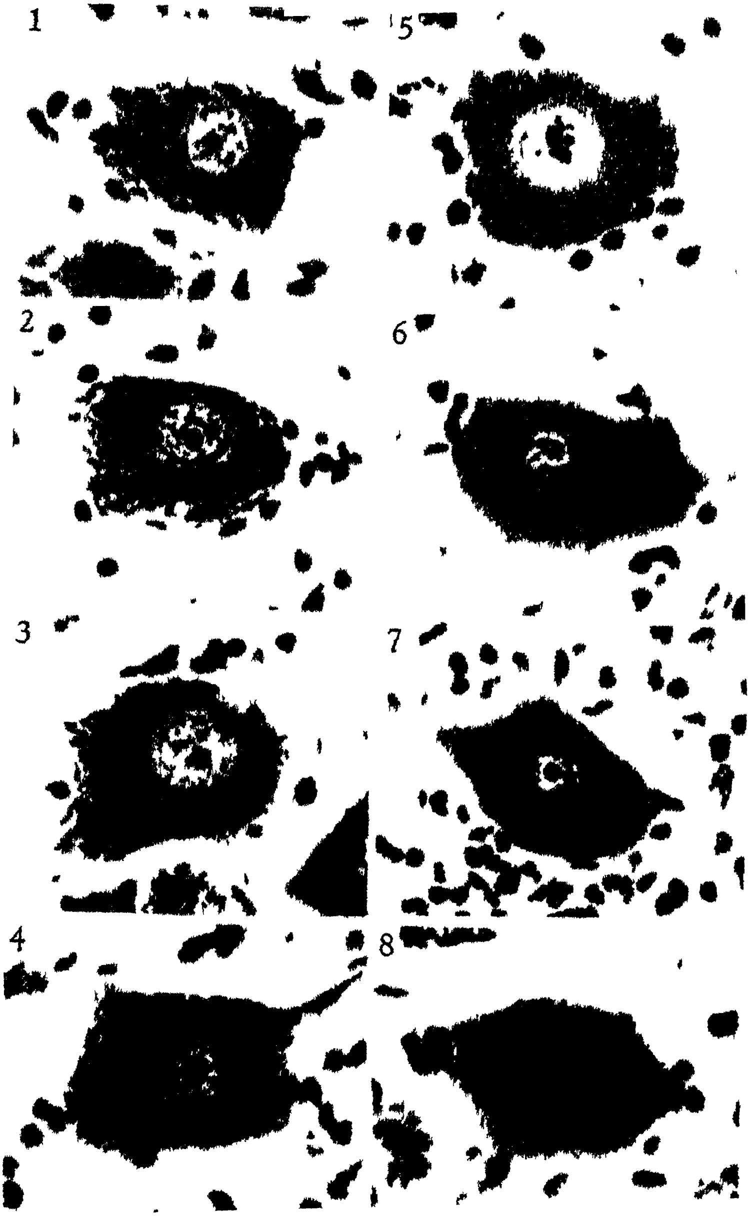
It has been tempting to ascribe the increased resistance of the regenerating nerve cells to changes induced in their nucleic acid metabolism, although other important changes also occur in these cells which might affect virus penetration or growth. The temptation arises from consideration of the

PLATE 3



For explanation see p 178

PLATE 4



For explanation see p. 178

following three points: (1) the nucleic acid metabolism of regenerating nerve cells is greatly altered; (2) the virus multiplication must depend at least in part on increase in virus nucleoprotein, either by new synthesis or by transformation of host materials; and (3) the earliest signs of virus activity in the nerve cells are seen histologically in the dissolution of Nissl substance. This depletion may progress to the point where cell necrosis occurs (Pl. 4). Although, unfortunately, we have not attempted as yet to follow the histological changes with a parallel study of ultra-violet absorption at 2650 A., it is probable that the reduction of Nissl substance is an indicator of depletion of nucleoprotein. Associated with these early changes in the nerve cells, which occur during the first day of infection, is a sharp rise in the concentration of virus in the infected tissue.

It is obvious that our present knowledge is far from indicating how virus growth is related to the nucleic acid metabolism of the nerve cell. Mere depletion of nucleoprotein in the cytoplasm of chromatolytic cells cannot account for their resistance to virus, since the greatest degree of resistance occurs in recovery stages of regenerating cells when reconstruction of Nissl bodies is well under way. Since Nissl bodies are, however, smaller than normal, and structurally not quite normal, until complete recovery occurs, it could be postulated that an important factor in the phenomenon of induced virus resistance in these cells is the disturbance of molecular arrangements in the complex nucleoprotein aggregate which we call the Nissl body.

There is a need for studies of the chemical constitution and structure of highly neurotropic viruses like that of poliomyelitis, and of the Nissl body itself. When the nucleoproteins of these substances are sufficiently characterized, we may have a better understanding of the growth of viruses in nerve cells, and of their high degree of host-cell specificity.

VI. DISCUSSION AND SUMMARY

It is interesting that all changes so far observed in the regenerating neuron, both morphological, physiological, and chemical, are in the direction of reversion toward the levels of the embryonic period, with subsequent return to adult levels as regeneration is completed. Both the developing and the regenerating neuron appear to have relatively low levels of cytochrome oxidase activity, low levels of accumulated cytoplasmic nucleoprotein, high levels of phosphate turn-over, and increased demands for protoplasmic synthesis. Unfortunately, adequate studies of O_2 uptake or glycolysis in regenerating neurons have not been made.

I now wish to conclude with a few more words of speculation and of recapitulation. The morphologist and the electro-physiologist often find it convenient to visualize the nerve cell as a relatively stable, if not rigid, system which serves as a connexion or a conduit between two distant points. This idea has its uses, but overlooks the possibility that the biggest job which the neuron may perform, in terms of biochemical work, is the job of continual replacement which is required to maintain its cytoplasmic dependency, the axon. It has been shown in many ways (see review of Needham, 1942, p. 523) that a cell which is carrying on its characteristic chemical activity, such as secretion, melanin production, or glycogen storage, does not divide, and conversely. Perhaps then it is the continual replacement of the axon, determined by the cell body, which precludes the mitosis of nerve cells after differentiation sets in. Perhaps this activity of the neuron is as characteristic as its work of conduction, and possibly the energy it requires is quantitatively greater. If this thesis holds, we must regard the embryonic outgrowth of the axon and the chemical activities which support it as processes which continue throughout life, as an axon replacement process. Regeneration after interruption of the adult axon can then be viewed not only as a reversion to embryonic activity but also as an artificially accelerated normal function. It seems clear that the nucleic acid metabolism of the cell-body cytoplasm plays an important role in these processes.

It is obvious that this field is wide open for further studies and that, in addition to the general problems of cell growth and differentiation, many practical problems such as the regeneration of injured nervous tissue and the action of neurotropic viruses or of neurotoxic drugs await the understanding which might come from such investigations.

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EXPLANATION OF PLATES

PLATE 1

Microscopic cross-section of lumbar spinal cord of rhesus monkey, stained with toluidine blue, and showing the distribution of the large motoneurons in the anterior grey columns (compare with Text-fig. 1). In addition to the cell bodies of the large motoneurons, the anterior columns contain many small neuroglia cells, blood vessels, and a feltwork of axons and dendrites not stained with this dye. The axons of the motoneurons on the right were severed 6 days before death, by spinal root section. 15μ section. ×35.

PLATE 2

Anterior grey columns from same section as shown in Pl. 1, but ×90. Note the proportion of motoneuron cell substance to other components of the grey matter (about 5%). (1) Normal side. Note the relative uniformity of size and of Nissl body distribution in the normal anterior horn cells, as compared with sensory ganglion cells (Pl. 3). (2) Note the relative uniformity of chromatolytic reaction in the cytoplasm of the motoneurons after spinal root section.

PLATE 3

Cells from sensory ganglia of a normal rabbit, stained with toluidin blue and illustrating the great variation in size and in Nissl body distribution in the cell population of these ganglia. Cells which show a chromatolytic pattern are seen in normal ganglia, so that experimental changes must be interpreted with caution. (1) Cells from a spinal ganglion. (2–7) Cells from the ganglion of the trigeminal nerve. (8–12) Cells from the nodose ganglion of the vagus nerve. Note the cells showing typical 'central chromatolysis' in (1) left centre, in (8) right and in (11) left. Note the heavy perinuclear aggregation of Nissl bodies in (3), (6), (9) and (10). Note the heavy central aggregation of Nissl bodies in (2), left, and the 'normal' pattern in the neighbouring cell. Note the disperse pattern of small Nissl bodies in (4), and the dense, heavy aggregations in (5) and (7).

PLATE 4

Regressive stages in rhesus motoneurons following infection with poliomyelitis virus. Gallocyanine stain. (1) Normal cell. The earliest visible changes are in the Nissl bodies (2) which are progressively reduced in size diffusely throughout the cytoplasm until no longer present (2–7). Nuclear changes are not seen until dissolution of cytoplasmic chromophilic material is well advanced (5–8).

TISSUE CHANGES IN MICE TREATED WITH PENTOSE NUCLEOTIDES

By L. D. PARSONS (London), J. M. GULLAND
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I. INTRODUCTION

Investigation into the biological action of the pentose nucleotides was undertaken in relation to tumour development in experimental mice. Certain changes occurring in the tissues of sarcoma-bearing animals suggested, as being worthy of study, the administration of compounds which act directly on the primitive undifferentiated cells of the reticulum, and experiments were made (Parsons, 1945) with a commercial mixture of 'pentnucleotides' as used clinically in cases of agranulocytosis. Since conflicting results were obtained, for example, in the splenic changes, and since there could be no justification for the use of a mixture of compounds whose individual effects were unknown, attention was turned to the pure crystalline nucleotides of yeast ribonucleic acid, adenylic, guanylic, cytidylic and uridylic acids. It was found that the systemic changes induced in mice by tumour growth were reproduced with singular exactness by the injection of these compounds into normal mice, and further that their administration to tumour-bearing mice affected the size of the tumours and modified some of the tissue changes characteristic of sarcoma growth. Some observations were also made linking the results of X-radiation of mice with those of administration of nucleotides.

The comparisons on which emphasis is laid are best made by a brief summary of the systemic effects of sarcoma growth in mice, followed by descriptions of the results of nucleotide administration to normal and tumour-bearing mice respectively.

It was found (Parsons, 1935, 1936, 1938, 1942, 1943; Mayneord & Parsons, 1937; Parsons & Warren, 1941) that during the latent period before tumour development in mice treated with carcinogenic compounds, abnormal cells appeared in the blood, a progressive rise in immature myeloid cells being a constant feature. A leucocytosis was not usually present. Eosinophil counts showed a high peak at variable points, but generally during the latter half of the latent period. Marked changes occurred in the lymph glands including proliferation of reticulum cells, atrophy of lymphoid tissue, plasmacytosis and deposits of material giving the prussian blue reaction histologically. A progressive increase in the total iron content up to fivefold the normal value was demonstrated (Warren, 1939). The number of giant cells per unit area in the spleen was decreased. Irradiation of the experimental mice, either previous to or during treatment with the carcinogenic compounds, both accelerated tumour formation and gave rise to a greater number of sarcomas.

In mice bearing primary sarcomas and in their grafted generations these tissue changes became rapidly accentuated. An increase in the total white-cell count occurred, which was often of a leukaemic type and more or less proportional to the rate of growth of the sarcoma. The normal relationship of polymorphs to lymphocytes was reversed, myeloid cells were numerous in the blood, and large pathological polynuclears with abnormal division of their nuclei appeared. Absolute values of lymphocytes were markedly less, and eosinophils tended to disappear. Splenic changes included reticulosis, increased deposits of material reacting iron-positive, deposits of black pigment not giving the prussian blue reaction, and myeloid metaplasia or amyloid infiltration. The giant cell count per unit area was decreased in spleens of mice bearing primary sarcomas, but increased in their grafted generations. Where myeloid metaplasia or amyloid infiltration had occurred in the spleen similar changes were frequently found also in the liver. Fatty degeneration and focal necrosis was noted in livers of mice bearing intraperitoneal grafted sarcomas. The changes in the lymph glands mentioned above were greatly accentuated, and myeloid and giant cells were found frequently throughout the lymphoid tissue. Plasmacytosis was a marked feature in many cases. Where a leukaemic blood condition accompanied the growth of the sarcoma, cellular changes were found in the bone marrow.

Such systemic changes, varying in their incidence with the strain of sarcoma and induced directly by the development of tumour tissue in the mouse, suggested that a substance or substances elaborated in the metabolic processes of the tumour cells and liberated in the body might be responsible for the widespread effects noted. The reticulosis, plasmacytosis and leukaemic blood condition in particular pointed to a profound stimulation of the primitive mesenchymal cells of the reticulum by these unknown substances originating in the local sarcoma growth.

II. MATERIAL AND METHODS

Mice. Stock and pure-line (C 57) mice were used in experiments to test the effect of injections of the nucleotides on normal mice. For experiments in tumour-bearing animals pure-line (C 57, CBA) mice were grafted with homologous methylcholanthrene sarcomas. Results of experiments with stock mice bearing heterologous tumours were inconsistent, and such experiments were therefore discontinued.

Tumours. The homologous methylcholanthrene sarcoma grafted in CBA mice and its tissue effects have already been described (Parsons, 1942). In the later generations this tumour tended to retrogress and the percentage of takes to fail. Thereafter only mice grafted with the C 57 methylcholanthrene sarcoma were used.

Compounds and dosage. The nucleotides were analytically pure crystalline samples prepared from yeast ribonucleic acid. The standard dose was 0·025 g. injected subcutaneously in the right flank five times weekly, either in solution in 3·2% disodium phosphate or as the water-soluble sodium salt; the use of the solvent did not modify the specific effects of the nucleotides. Such relatively large doses were adopted in order to accelerate the appearance of, or accentuate, the biological effects, but occasionally smaller doses were given over short periods if the condition of the mouse seemed to warrant this change. Where two of the nucleotides were injected simultaneously the dose of each was halved.

X-radiation. The mice were confined in a wooden box 15·2 × 15·2 × 3·8 cm. (inside measurements), the shallowness of the space preventing overlying and allowing equal distribution of irradiation. A constant potential generator was used, operating at 180 kV. and 4 mA. with a filter of 0·5 mm. copper and 0·2 mm. aluminium. The focal distance was 40 cm. Total dosage (one exposure), and varying with the different groups of mice treated, was 250–500 r. at rate of 30·7 r./min. with back scatter.

III. NORMAL MICE TREATED WITH PENTOSE NUCLEOTIDES

Prolonged treatment of normal mice was carried out over periods varying from 2 to 5 months. Young mice treated with adenylic acid showed severe shock on injection and considerable wasting during treatment, but older mice exhibited little or no wasting. Mortality was higher in mice treated with adenylic acid than in those injected with the other nucleotides. Local inflammatory changes occurred at the site of injection, which, in mice treated with cytidylic acid, were accompanied by the production of glairy yellow mucoid material, similar to that seen round a developing sarcoma in the malignant phase. In these cases histological examination of the tissue gave a marked prussian blue reaction, which reaction was less intense in the tissues at the site of injection in mice treated with guanylic or adenylic acid.

Blood. Mice treated with adenylic or cytidylic acid showed a considerable rise in the total white-cell count within 24 hr. of the initial injection, and this leucocytosis was usually maintained with some fluctuations throughout treatment; values as high as 58,000 w.b.c./c.mm. were recorded. Absolute values of lymphocytes, polymorphs and immature myeloid cells were increased, and crossing of the lymphocyte line by the polymorph was frequent except in pure-line C 57 mice injected with cytidylic acid, where the polymorph count was consistently low. A rise in the eosinophil count was rare and never exceeded 6%.

Mice treated with guanylic acid showed a slight initial rise or fall in the total white-cell count within 24 hr. of the first injection, and successive doses failed to cause more than a moderate leucocytosis. Crossing of the lymphocyte line by the polymorph occurred occasionally. A considerable rise in the number of eosinophils was a marked feature occurring at frequent intervals and often totalling 12–16% of the total count. In normal mouse blood the proportion of these cells rarely exceeds 1–3%.

Spleen. Amyloid infiltration of the spleen was noted in X-radiated and non-irradiated mice treated with adenylic or guanylic acid, but not in the spleens of mice treated with cytidylic acid. Five of the twelve irradiated mice treated with guanylic acid showed this change—a percentage higher than that obtaining in non-irradiated mice, where only three cases occurred among the twenty mice injected. Four of thirty mice injected with adenylic acid developed amyloid infiltration.

Heavy deposits of iron-reacting material and of pigment reacting iron-negative were found in spleens of mice treated with the three nucleotides, being most marked in those injected with adenylic or guanylic acid. The dorsal half of the spleen of a young mouse treated with guanylic acid and killed on the 69th day showed a prominent black nodule similar to those seen in spleens of C 57 tumour-bearing mice or mice grafted intraperitoneally with the malignant sarcoma. Reticulosis and plasmacytosis were fairly constant features in spleens of mice injected with the three nucleotides. Lymphoid tissue was usually much diminished. In some cases immature myeloid cells of the ring type occurred in large numbers. The numbers of giant cells per unit area were found to be decreased in spleens of mice treated with adenylic acid, to be slightly above the normal count in those injected with cytidylic acid, but to be markedly increased in spleens of mice treated with guanylic acid. Irradiation was found to accentuate these differences. Spleens of mice injected simultaneously with adenylic and guanylic acids showed pyknosis of giant cells and lymphoid tissue, and focal necrosis was common—more especially in irradiated mice. Such spleens stained with Congo red did not give the typical amyloid reaction. Focal necrosis of the spleen and liver is common in mice subjected to heavy doses of X-radiation, and very common in mice bearing intraperitoneal grafted sarcomas (Parsons & Warren, 1941). Injection of the two purine compounds simultaneously appears to exert an effect similar to that induced by X-radiation or the growth of a tumour.

Liver. Amyloid infiltration was found in the livers of mice where a similar condition occurred in the spleen, and periportal infiltration with round cells was common. Proliferation of the Kupfer cells was frequently noted, an dareas of focal necrosis giving a faint pink colour with Congo red, but not typical of amyloid, were observed in livers of mice treated with the two purines simultaneously.

Lymph glands. Lymph nodes of mice treated with each of the three nucleotides were either normal, enlarged or diminished in size. The tissue changes noted were similar to those described (Parsons, 1938) in glands of irradiated mice, in mice undergoing treatment with carcinogenic compounds, or bearing primary or grafted sarcomas. Histologically marked proliferation of the fixed reticulum cells was noted with widening of the sinuses, which often contained free macrophages laden with iron-reacting material. Diminution of lymphoid tissue was usual. Deposition of iron-reacting material occurred commonly at the periphery of the gland, where the littoral cells formed broad bands often running down into the medulla. Mast cells varying in size were found in large numbers in those areas where the prussian blue reaction was pronounced. Isolated giant cells with single or multilobulated nuclei were occasionally found scattered through the lymphoid tissue. In many cases plasma cells were abundant and showed mitotic figures and twin nuclei. Pyknosis of lymphocytes in the cortical nodules was sometimes noticed and was most marked in lymph nodes of irradiated mice treated with adenylic and guanylic acids simultaneously. In these, small areas of focal necrosis also occurred at the periphery of the gland.

Lymph glands stained with Pappenheim showed deep staining of the fixed reticulum cells in mice treated with adenylic and guanylic acids simultaneously, though this occurred occasionally in glands of mice injected with any of the three nucleotides. Such cells are usually distinguished with difficulty owing to their faint reaction to the dye. The action of the nucleotides appears to stimulate proliferation of these cells, which become greatly enlarged and exhibit refractile droplets in their interior. In glands of irradiated mice treated with the two purines simultaneously the large size and intensely red colour of these cells gave a remarkable appearance. Many of gigantic size and showing active phagocytosis of red blood cells, lymphocytes, granulocytes and plasma cells were free in the sinuses, but similar cells in all stages of development could be distinguished still attached to the fixed reticulum cells of the stroma. Their pale-staining nuclei were in many cases of small size and situated centrally. Multinucleated giant cells were rarely seen in these tissues. Considerable iron-reacting deposits were demonstrated in many of these enlarged reticulum cells.

IV. TUMOUR-BEARING MICE TREATED WITH PENTOSE NUCLEOTIDES

One hundred and sixty-one mice bearing homologous methylcholanthrene grafted sarcomas were injected subcutaneously in the left flank with the individual nucleotides in disodium phosphate five times weekly. Of these fifty-one were treated with adenylic acid, fifty-four with guanylic acid, thirty with cytidylic acid and twelve with uridylic acid. Nine mice received adenylic and guanylic acids simultaneously, and five irradiated mice were injected with guanylic acid only. Treatment was begun on the 1st, 4th or 6th day from the date of grafting, and both treated and control mice were killed at varying intervals from the 12th to the 21st day from start of the experiment. The individual tumours were immediately excised and weighed.

Mice injected with adenylic or guanylic acid showed considerable reduction in tumour growth. The total weight of the tumours in mice treated with adenylic acid was 179·6 g. as compared with 291·8 g. in the controls, whereas in those treated with guanylic acid the total tumour weight was 149 g. against 244·7 g. in the controls. Cytidylic acid appeared to have little or no effect on tumour growth, the total tumour weight in the treated mice being 152 g. as compared with 157·3 g. in the controls. The action of uridylic acid on tumour development appeared to be acceleratory, the total tumour weight in the small number of twelve mice treated being 93·1 g. as compared with 74·9 g. in the controls.

Statistical analysis has shown that the differences in tumour weights for experimental and control animals are significant in the case of adenylic and guanylic acids, but not significant for cytidylic acid.

The simultaneous injection of adenylic and guanylic acids appeared to increase their inhibitory action, the total weight of tumours in the nine treated mice being 21·9 g. as against 52·9 g. in the controls.

General irradiation has been found to promote tumour growth in experimental animals (Mayneord & Parsons, 1937), and it might be expected that the effect of an inhibitory compound would be lessened in mice irradiated previous to grafting and injection of the nucleotides. This expectation was confirmed in the small number of mice so treated. In the group of five mice irradiated with 450 r. and injected with guanylic acid the total weight of tumours was 22·3 g., that of the controls 29·8 g. These figures give an average diminution in tumour growth in the irradiated mice of 1·5 g. as compared with the controls, whereas in non-irradiated mice similarly treated the average decrease in the sarcomas was 2·3 g.

Examination of splenic changes accompanying the growth of the sarcomas showed that the spleens of mice treated with adenylic or cytidylic acid were decreased in weight as compared with the controls, and the number of giant cells per unit area markedly lessened. Mice injected with guanylic or uridylic acid showed increase of splenic weight; both caused a considerable increase of giant cells per unit area, the former being the more active in this respect.

Deposits reacting iron-positive were observed in the spleens and lymph glands of untreated C 57 grafted mice, and their amounts appeared to be increased by injection of adenylic acid, decreased by guanylic and uridylic acids, and to be unchanged after treatment with cytidylic acid. As no quantitative estimations have yet been made of the total iron content of these tissues, no exact opinion can be formed on this point.

The bloods of C 57 tumour-bearing mice treated with adenylic or guanylic acid showed a rather lower total white blood cells count than the untreated controls in which a moderate leucocytosis was present. Since retrogression of a sarcoma has been shown (Parsons, 1936) to be accompanied by a corresponding fall in the number of white blood cells, it would be expected that inhibition of tumour development by the action of the purine compounds would also cause a lowered blood count.

V. DISCUSSION

The singular similarity in the systemic changes induced in normal mice injected with the pentose nucleotides to those observed in mice undergoing treatment with carcinogenic compounds, or bearing primary or grafted sarcomas, suggests that:

(*a*) the four nucleotides, adenylic, guanylic, cytidylic and uridylic acids, or similarly constituted compounds, may play a part according to their specific properties in the local and systemic changes occurring during the latent or pre-cancerous period before tumour development;

(*b*) similar substances originating in a developing sarcoma may be responsible for the varying phases in tumour growth and the coincident systemic effects;

(*c*) if pentose nucleotides are liberated in tumour metabolism and are factors in the tissue changes, these would vary with the amounts of the different nucleotides set free and according to their specific action. This possibility might have a bearing on the peculiar characteristics of different sarcomas, the varying incidence of the tissue changes, the rapidity or slowness of the sarcoma growth, and the degree of malignancy.

These suggestions lead naturally to the following speculations:

(*d*) if, as has been shown by the preliminary experiments on tumour-bearing mice, the pentose nucleotides possess growth-inhibiting or growth-promoting properties, it is possible that their mutual actions maintain normally a balance in cell growth, repair and multiplication. Any disturbance of such a balance leading to excess of growth-promoting substances might be an initial factor in starting neoplastic changes;

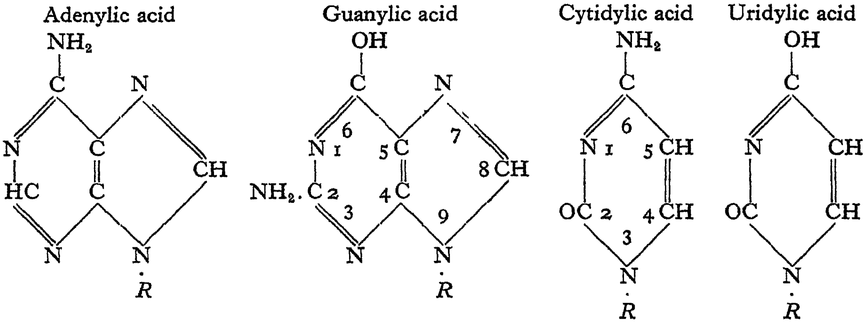
(*e*) if an excess of pentose nucleotides is liberated during tumour metabolism, as is now suggested by the correlation between their administration and sarcoma growth, abnormally high concentrations of these substances would be available for the synthesis of polynucleotide-protein complexes, whether specific for the tumour or not.

Evidence in favour of such possibilities is not lacking. Nucleic acids and their derivatives are known to play a major part in relation to tissue growth (Davidson & Waymouth, 1944, 1945), embryonic development (Caspersson & Thorell, 1941*a*, *b*; Davidson & Waymouth, 1944*a*, *b*) and neoplastic growth (Caspersson & Santesson, 1942; Davidson & Waymouth, 1944*b*, *c*). In general, it has been found that a high content of nucleic acid characterizes rapidly growing tissues including tumours, although in the latter it appears to depend inversely on the amount of fibrous tissue (Davidson & Waymouth, 1944*c*). The presence of the pentose nucleic acids or nucleotides in considerable concentrations in the cytoplasm of tumour cells is indicated by the work of Caspersson, Nyström & Santesson (1941), Mitchell (1942, 1943), Davidson & Waymouth (1944*c*), and Claude (1939, 1940).

The work of Mitchell (1942, 1943) suggests that an accumulation of nucleotides occurs in the cytoplasm of rapidly growing cells after X- or γ-irradiation. Generally irradiated mice show many of the systemic effects induced by tumour growth or the action of the pentose nucleotides. It seems possible that the effect of general irradiation is the liberation of an excess of nucleotides, which induce tissue changes similar to those noted in mice treated with carcinogenic compounds or the nucleotides. This excess may have a bearing on the greater incidence of sarcomas in a shorter time in irradiated experimental mice as compared with non-irradiated. It has also been claimed that ultra-violet irradiation results in the release from living cells of pentose nucleic acid derivatives into the intracellular fluids (Loofbourow, 1942*a*, *b*), and if a release of pentose nucleotides can be regarded as a response to cell injury, the liberation of these compounds might explain to some extent the tissue changes occurring in mice during the latent period before tumour formation. In tissues of mice treated with carcinogenic compounds deposits of these substances remain locally at the site of injection, undergoing gradual absorption and inducing inflammatory changes in the tissues around. If accumulations of the different nucleotides are formed locally, it is possible that some of their excess content may be liberated in the body and effect those changes so exactly reproduced in normal mice by injection with similar nucleotides. If the actions of the individual nucleotides vary according to their inhibitory or growth-promoting activity, an excess of one or the other might retard or accelerate tumour formation. This might explain the great variation in the duration of the latent period seen in experimental mice.

Variations in the growth and malignancy of sarcomas are difficult to explain without reference to the pentose nucleotides. For, should the four nucleotides derived from an excess of nucleic acids known to exist in tumour cells as compared with the normal tissues of origin possess the properties of stimulating or retarding cell multiplication, fluctuations would occur in the growth and malignancy of the sarcoma in proportion to the amount of one or more of the nucleotides metabolized. Caspersson & Santesson (1942) state that marked variations in the nucleic acid content may occur in different regions of malignant growths with high concentrations in the cytoplasm of the tumour cells. Caspersson further suggests (1936, 1941*a*, *b*) that this high concentration is associated with rapid protein synthesis. It has been shown also that in rapidly growing tumour tissue the concentration of nucleotides is lower than in normal tissue, whence it is concluded (Davidson & Waymouth, 1944*a*, *c*; 1944–5) that the cytoplasm accumulates pentose polynucleotides rather than simple nucleotides; it may therefore be deduced that rapid polynucleotide synthesis occurs and that the non-polymerized nucleotides diffuse quickly from the cell, as might be expected. The tumour cell is thus well endowed with materials for the synthesis of nucleoprotein, and pushing speculation further, if such nucleoprotein were specific for the tumour and part of a phospholipin-nucleoprotein complex, such as have already been isolated in the case of certain viruses (Claude, 1938, 1939, 1940; Taylor, Beard, Sharp & Beard, 1942), its synthesis at a threshold concentration of the components might explain the sudden appearance and development of sarcomas in experimental mice after prolonged and varying latent periods.

The picture obtained from the results of the preliminary observations on the action of the pentose nucleotides suggests that these intimate constituents of the cell may be continually exerting either a similar or opposing action to each other—associated at one time in the production of similar tissue changes, dissociated at another to exert their own peculiar effect. Since similar and dissimilar effects produced by the action of the four nucleotides on the tissues must depend on their molecular structure an examination of the formulae of the nitrogenous radicals reveals suggestive relationships, which may be purely fortuitous and have no significance, but are being further investigated.



R = Ribose phosphate.

From the preceding formulae it will be seen that:

(*a*) the pyrimidine ring of adenine is in a lower state of oxidation than in the other radicals, and adenylic acid induces a condition of shock on injection, a property not possessed by the other compounds;

(*b*) the group $N{=}C.NH_2$ occurs in the same position (1 : 6) in both adenylic and cytidylic acids, and these compounds exert similar effects on the blood and spleen;

(*c*) the group $N{=}C.OH$ is similarly placed (1 : 6) in the structures of guanylic and uridylic acids, a variation from the group $N{=}C.NH_2$ mentioned in (*c*), and these acids have a similar effect on the spleens of tumour-bearing mice, different from that of adenylic and cytidylic acids;

(*d*) guanylic acid alone has an amino group at position 2, and it alone has the particular effect of stimulating the production of eosinophil cells;

(*e*) the amino group occurs in position 6 in adenylic and cytidylic acids, and both compounds increase the number of myeloid cells in the blood, but do not affect the eosinophil count. They also cause a considerable leucocytosis, whereas guanylic acid induces only a moderate increase in the white blood cell count.

VI. SUMMARY

1. Preliminary experiments to test the action of the pentose nucleotides on tumour-bearing mice have shown that the purine compounds, adenylic and guanylic acids, cause inhibition of tumour growth. Of the pyrimidine nucleotides cytidylic acid appears to have little effect on the rate of tumour development, whereas uridylic acid has a growth-promoting action.

2. Examination of splenic changes in tumour-bearing mice treated with the nucleotides has shown that adenylic and cytidylic acids decrease both

the size and weight of the spleen and the number of giant cells per unit area. Guanylic and uridylic acids appear to exert an opposite effect.

3. The combined actions of the pentose nucleotides on the tissues of normal mice have been found to reproduce with singular exactness the systemic effects induced in mice by X-radiation, by treatment with carcinogenic compounds, or by the growth of a primary or grafted sarcoma.

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HISTOCHEMICAL OBSERVATIONS ON NUCLEIC ACIDS IN HOMOLOGOUS NORMAL AND NEOPLASTIC TISSUES*†

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I. INTRODUCTION

Histochemical and cytochemical methods of analysis permit the study of the constituent components of tissues and of cells and the correlation of chemical and morphologic data. Such information is of especial value when expressed in quantitative terms. The increasing variety of available methods permits one to apply microchemical analysis to small parts of tissue (Linderstrøm-Lang, 1943; Holter, 1943; Norberg, 1942; Cowdry, 1945), or to fragments of cells such as whole nuclei (Dounce, 1943), or nuclear or cytoplasmic constituents (Claude & Potter, 1943; Claude, 1946*a*, *b*). Such techniques often permit the adaptation of accurate chemical methods, but do not frequently allow detailed morphologic studies, since the methods usually destroy the organization of the tissue. The pioneer work of Caspersson and his coworkers (Caspersson, 1946) has shown that certain physical properties of cells can be measured quantitatively by ultra-violet light on intact living or fixed tissues. Another method which is less quantitative, but of potentially wide application, is based upon the absorption of visible light by pigments within tissue. Of more importance for such measurements than endogenous pigments are those formed within the tissues by specific physicochemical staining reactions. Although relatively little research has been done with such colorimetric chemical analyses of cells, there is an increasing interest in these histochemical techniques (Gomori, 1945) and in their quantitative photometric estimation (Stowell, 1945*b*).

This paper will summarize the results of some of the quantitative photometric histochemical observations made during the past six years on nucleic acids in normal and neoplastic tissues.

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† This article covers only part of the talk given by Dr Stowell. Other parts, dealing with the Feulgen and ribonuclease techniques, have already been published (Stowell, 1945*c*, 1946*b*, and Stowell & Zorzoli, in press).

II. HISTOCHEMICAL METHODS

The Feulgen technique, as employed in this photometric work, has been modified slightly (Stowell, 1945*c*) to permit more accurate control of the reaction for purposes of photometric estimation. Rather than attempting to express the results in terms of highly quantitative units, comparative data have been derived in which the amounts of thymonucleic acid in one tissue is indicated in terms of the amount in other tissues which have been treated under comparable conditions. Usually the types of tissue being compared were parts of the same block and section and therefore received nearly identical conditions of fixation, dehydration, embedding, sectioning and staining. These technical procedures were carefully standardized and controlled. Several sections were mounted on the same slide and all the slides were stained simultaneously in the same dish.

Ribonuclease. Despite awareness of the limitations and unproved specificity of ribonuclease, an attempt was made nevertheless to get useful information on the changes in ribonucleic acid in a series of human tumours as compared with their homologous normal tissues (Stowell, 1946*a*). The technical details will not be repeated here, but some of the results will be summarized with the presentation of the measurements on tumours.

Desoxyribonuclease. Experiments employing desoxyribonuclease have been mentioned elsewhere (Stowell, 1946*b*), but because of their necessarily somewhat preliminary nature will not be described in detail. Most of the results were disappointing, since only in a few instances was a selective loss of Feulgen-staining properties obtained. For this enzyme also, a great deal of fundamental work is needed to establish the optimum conditions for use and to evaluate the specificity of action upon tissues.

III. PHOTOMETRIC APPARATUS AND METHODS

Photometric instrument. The microphotometric instrument for measuring the absorption of visible light in tissues consists of a stable light source, a microscope, a photocell and amplification and recording apparatus. Details of the instrument have been published (Stowell, 1942).

In the operation of the instrument, any shift in the amplifier, change in the light source or inherent differences in light transmission through the slide, cover-glass or mounting media are corrected by frequently determining the transmission of light through areas of the slide not containing tissue which are adjacent to parts of the tissue being measured.

Cellular measurements. One of the advantages of the photometric method is that it permits the correlation of the results with morphologic data. The importance of the relation of quantitative measurements to the living biologic unit, the cell, is overlooked by many physiological chemists. With the method described the measurements of mean amounts of substances per unit volume and per cell can be further related to mean volumetric measurements of the cell, cytoplasm, nucleus, nucleolus, chromatin and nuclear sap. This is accomplished by means of the method of determining volumetric ratios suggested by Chalkley (1943). Although experiments testing the accuracy of some volumetric methods showed that they might have an error as large as 45% (Stowell & Cooper, 1945), the method of Chalkley is more accurate and rapid. For instance, in this work with tumours to be described, the mean cell size was arbitrarily determined by dividing a figure for the unit volume of the tissue by the mean number of cells in each field measured. For example, in normal human liver the arbitrary mean cell volume was 16·6, and of the 3300 foci, 246 were deleted because they were on empty spaces such as the lumen of blood vessels, 2678 were on cytoplasm, 229 on chromatin, 95 on nuclear sap and 52 on nucleoli, making a total of 376 on the hepatic cell nuclei. These figures indicate that of the mean total cell volume there was 87·7% cytoplasm, 7·5% chromatin, 3·1% nuclear sap and 1·7% nucleolus or 12·3% nucleus. From the percentile figures and mean cell size, arbitrary values for the volumes of the different cellular constituents can be calculated for comparison with similar measurements on other tissues and for correlation with the absorption measurements. Only the nuclear-cytoplasmic ratio was calculated in specimens in which the chromatin was dense or the nucleolus indistinct.

Evaluation of technique. The photometric measurement of pigments in tissues has certain inherent difficulties which tend to limit the accuracy of the method. If the concentration of pigment is great, then small changes are not readily detected in thick, inhomogeneous tissues. The adsorption of pigment on to cellular interfaces would also produce errors in quantitative measurements. Although there is some deviation from Lambert-Beer's law, and the method is not as highly quantitative as the ultra-violet techniques by Caspersson (1946), nevertheless, useful information can be obtained and the errors minimized by comparing the relative values in different tissues rather than attempting to express them in more exact quantitative units. The results are readily reproducible and agree with macrochemical analyses.

The chemical analyst must remember that most tissues should not be treated as homogeneous masses of uniform consistency, since they are made up of varying amounts of different types of cells, of intracellular substances, and of vascular tissue. The adjacent parenchymal cells of an organ or tumour, as well as the individual parts of the cells, vary greatly in their chemical composition, function and morphology. As compared with macrochemical methods, the photometric technique has the advantage that it permits the visualization of the cells during the measurements instead of destroying them. Thus measurements are made on an identified sample from which important artifacts such as foreign material, inflammatory or extraneous cells and necrotic tissues may be recognized and deleted from the measurements. The results are readily correlated with morphologic cellular data. There are obvious advantages of objective, accurate measurements (which can be analysed statistically) with a sensitive photocell over less accurate and opinionated estimations with the human eye. Statistical analysis of data in these photometric histochemical methods is indicated because the size of the sample of cells is small compared with that employed in macrochemical methods. The usefulness of colorimetric histochemical techniques will increase with advances in chemical colorimetry and histochemistry and cytochemistry. Because of its inherent limitations and advantages this photometric histochemical method, as well as most other histochemical and cytochemical techniques, is best employed as one of several methods of obtaining data which can be used to supplement and check each other.

IV. OBSERVATIONS ON TUMOURS

This photometric histochemical method of studying thymonucleic acid has been applied to (1) a comparison of normal epidermis, hyperplastic epidermis and carcinomas induced in the skin of mice by methylcholanthrene (Stowell, 1942); (2) a study of the effects of X-rays upon the thymonucleic acid content of transplantable mammary tumours in rats and mice (Stowell, 1945*a*); (3) a comparison of normal, hyperplastic and malignant cells of human epidermis (Stowell & Cooper, 1945); and (4) a study of nucleic acids in twenty different human tumours (Stowell, 1946*a*). The initial observations on nucleic acid in epidermoid carcinomas induced by methylcholanthrene showed that, as compared with normal epidermis, the amounts of thymonucleic acid per unit volume were less in many specimens of hyperplastic epidermis, a difference which was attributed to the greater amounts of cytoplasm in hyperplastic tissues. The benign papillomas and malignant carcinomas showed great differences in their thymonucleic acid content per unit volume of tissue, although in some instances it was materially increased. The cells of some of the carcinomas contained increased mean amounts of thymonucleic acid.

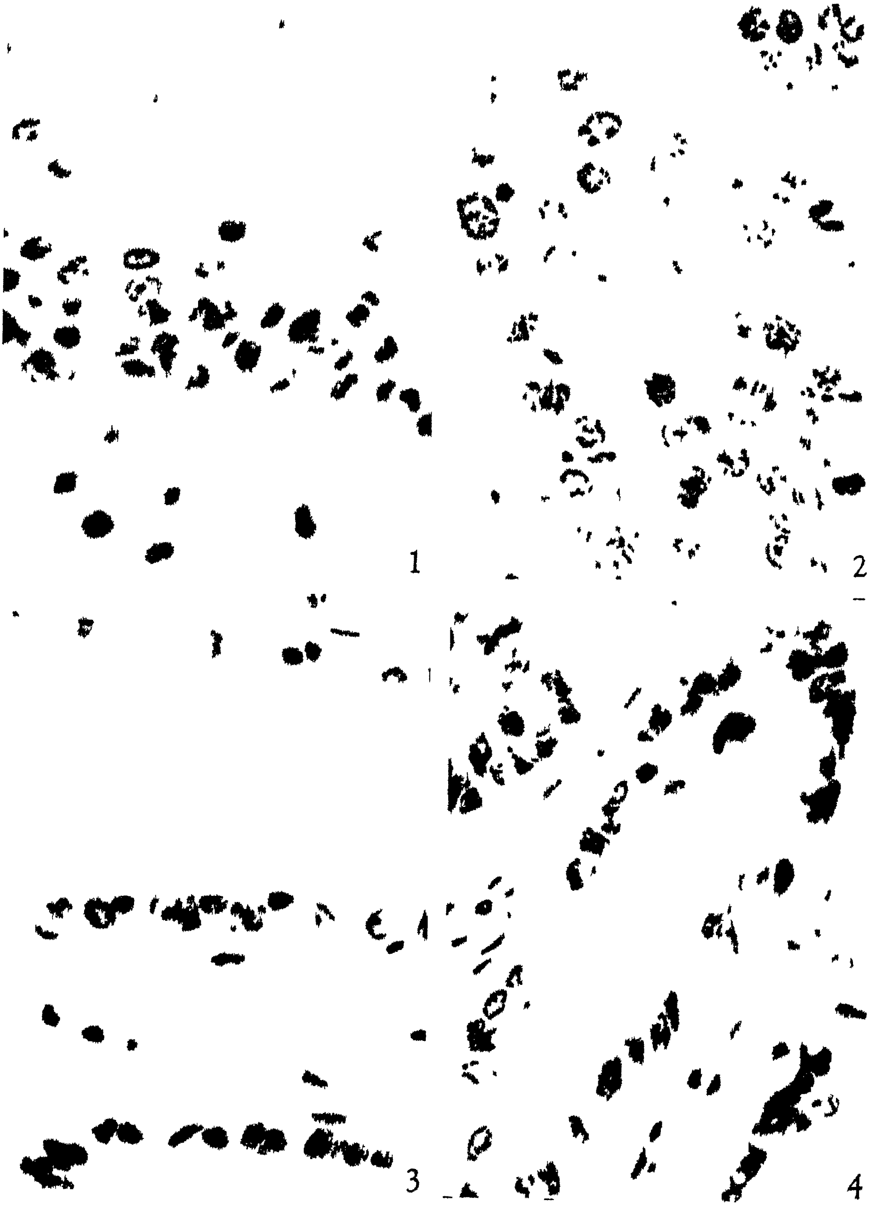
That röntgen rays produce a decrease in the thymonucleic acid of tumours is suggested by the finding that in six mammary tumours in rats which received 4000 r. all showed a significant decrease in the amount of thymonucleic acid per unit volume of tissue and two had a significant decrease in the amount per cell. Tumours which received inadequate doses of 2000 r. gave variable results. Of the sixteen mammary tumours in mice, after irradiation all showed a decrease in the thymonucleic acid per unit volume of tissue, in nine the decrease was statistically significant. The thymonucleic acid per cell decreased in twelve tumours and was significantly decreased in four. Both of these transplantable mammary tumours were somewhat resistant to radiation, and it is possible that more radiosensitive tumours would give more striking results. Additional studies with more radiosensitive tumours in animals, however, have been interrupted. Unpublished observations on a few human tumours have also shown that röntgen radiation produces a decrease in the thymonucleic acid in some tumours, but little effect with small doses on resistant tumours. This work should be confirmed with more suitable material and correlated with detailed morphologic cellular data.

The observations to be summarized here in more detail are those which have been made on human tumours. The data on thirty-one specimens, which have already been presented in two separate papers (Stowell & Cooper, 1945; Stowell, 1946*a*), will be combined and correlated to ascertain the status of the working hypothesis that there is a fundamental primary disturbance in the nucleic acid and nucleoproteins in tumours (Stowell, 1945*d*).

The material in the study may be divided into two main groups consisting of sixteen epidermoid carcinomas and fifteen miscellaneous tumours. Carcinoma specimens nos. 1–12 were from various sites on the external body surface; no. 13 was from the epiglottis and nos. 14–16 were from the larynx. The degree of anaplasia of each carcinoma, designated as grades I–IV, is indicated in Table 1. The second group of specimens includes: two transitional cell carcinomas, one from the nasopharynx (no. 17) and the other (no. 18) from the urinary bladder; a basal cell carcinoma; a turban tumour of the scalp; a renal cell carcinoma; eight adenocarcinomas, including examples from the prostate, stomach (Pl. 1, fig. 4), colon, rectum and bronchus; and a leiomyosarcoma. The thirty-one specimens for analysis were selected from a large number of surgical and autopsy specimens because they showed negligible artifacts and represented a suitable variety of tumours. The material was obtained at surgical operations and fixed promptly or was from autopsies performed less than two hours post-mortem. To facilitate the accuracy of comparisons, specimens were used in which the normal and neoplastic tissues were in close proximity so that measurements could be made on adjacent parts of the same section. The tissues were prepared and stained with meticulous care to give uniform results. Pl. 1, figs. 1 and 2 illustrate areas from the stratified squamous epithelium of the

PLATE 1

to face p 195



For explanation see p. 206

larynx and epidermoid carcinoma (no. 14) while normal gastric mucosa and adjacent adenocarcinoma of the stomach are shown in Pl. 1, figs. 3 and 4.

The specimens were prepared in a standard manner and photometric measurements made upon fifty areas of two adjacent serial sections which had been stained by the Feulgen technique. Whenever possible measurements were made on adjoining normal epidermis, hyperplastic epidermis and epidermoid carcinoma. However, for specimens nos. 6–10 only hyperplastic and no normal tissue was available for comparison and for nos. 11–16 no suitable hyperplastic tissue was present. To conserve space the detailed data of absorption and cellular measurements are not repeated, and only the ratios obtained by dividing the mean absorption for the carcinoma by the absorption for the normal or hyperplastic tissue are given in Table 1. The statistical significance of the differences between the neoplastic and control tissue for absorptions per unit volume and per cell are indicated as values for P, the probability. Values of P greater than 0·01 indicate less than one chance in a hundred of obtaining as great differences by accident and are considered statistically significant values. Negative values for P indicate the chances of getting greater absorption in the normal tissue.

Table 1. *Relative thymonucleic acid content per unit volume and per cell of epidermoid carcinomas expressed as ratios of mean absorption of tumour to normal epidermis,* T/N; *and tumour to hyperplastic epidermis,* T/H

No. and grade of tumour	Absorption per volume				Absorption per cell			
	T/N	P	T/H	P	T/N	P	T/H	P
1–III	1·32	0·000	1·54	0·000	1·31	0·000	1·49	0·000
2–II	1·14	0·007	1·46	0·000	1·26	0·000	1·08	0·097
3–II	1·00	0·464	1·48	0·000	0·82	−0·012	1·04	−0·125
4–II	0·96	−0·104	1·48	0·000	1·22	0·000	1·16	0·000
5–I	1·09	0·065	1·32	0·000	1·03	0·095	0·99	−0·136
6–I	—	—	1·60	0·000	—	—	1·07	0·469
7–III	—	—	1·38	0·000	—	—	1·12	0·000
8–III	—	—	1·30	0·000	—	—	1·14	0·087
9–III	—	—	1·28	0·000	—	—	1·05	0·046
10–I	—	—	1·77	0·000	—	—	1·48	0·000
11–III	1·38	0·000	—	—	0·96	−0·048	—	—
12–II	1·47	0·002	—	—	1·50	0·000	—	—
13–II	2·18	0·000	—	—	1·08	0·230	—	—
14–III	2·18	0·000	—	—	1·16	0·000	—	—
15–III	1·13	0·436	—	—	1·19	0·136	—	—
16–II	1·42	0·003	—	—	1·09	0·248	—	—

Of the eleven epidermoid carcinomas in which comparisons with normal tissues could be made, nine tumours had more thymonucleic acid per unit volume and per cell. The amount was significantly greater per unit volume in seven and per cell in five. The ten comparisons of hyperplastic epithelium and carcinoma showed that in each instance there was more thymonucleic acid per unit volume in the tumours, and in nine of the ten there was more per cell. These differences in the amounts per unit volume were statistically significant in 100% of the instances and per cell in 40%.

Similar ratios for the other specimens, comparing the percentages absorption per unit volume and per cell and the statistical validity of the differences between the tumour and homologous normal tissues, are given in Table 2. Of these fifteen specimens the amounts of thymonucleic acid per unit volume of tissue and per cell were increased in thirteen instances and significantly so in seven. Of the thirty-one tumours reported here and in three other unreported instances, making a total of thirty-four specimens, no tumour has contained a statistically significantly decreased amount of thymonucleic acid per unit volume or per cell.

Table 2. *Ratios of thymonucleic acid content per unit volume and per cell in tumour* (T) *and normal* (N) *tissues*

Tumour	Per volume		Per cell	
	T/N	P	T/N	P
17. Transitional cell	1·26	0·012	0·92	−0·203
18. Transitional cell	1·16	0·147	1·90	0·002
19. Basal cell	1·34	0·023	0·95	−0·341
20. Turban tumour	1·56	0·000	1·29	0·014
21. Renal cell	1·16	0·063	1·10	0·168
22. Prostate	0·78	−0·018	1·58	0·301
23. Stomach	2·09	0·000	1·82	0·000
24. Colon	1·01	0·221	1·13	0·464
25. Colon	1·38	0·001	1·54	0·001
26. Rectum	2·18	0·000	1·77	0·000
27. Rectum	2·28	0·000	1·35	0·029
28. Bronchus	1·05	0·367	1·53	0·004
29. Bronchus	0·98	−0·409	1·86	0·000
30. Liver cell	1·98	0·000	1·68	0·000
31. Leiomyosarcoma	2·01	0·000	1·07	0·359

Measurements of ribonucleic acid content as indicated by ratios of decreased pyronin staining following treatment with crystalline ribonuclease are summarized for some of the specimens in Table 3. The absorption data were obtained from four serial sections adjacent to those used for the Feulgen staining. Measurements were not attempted on some of the specimens, on others technical difficulties interfered with results, in seven instances there were no appreciable differences between the absorption of normal and neoplastic tissues; hence, data are only presented on nine tumours. The results are not considered highly quantitative, but of six tumours showing increased amounts of ribonucleic acid per unit volume, in four the magnitude of increase was at least 50%. Of the seven tumours having increased amounts per cell, the change was greater than 50% in four.

The result of the cellular measurements of the tumours (Stowell &

Cooper, 1945; Stowell, 1946*a*) is briefly summarized in Table 4. In this table values for tumour tissue significantly increased or decreased over those

Table 3. *Ratios of ribonucleic acid content as indicated by the relative reduction in staining with pyronin of normal* (N) *and tumour* (T) *tissues after treatment with ribonuclease*

Specimen	Ratios T/N	
	Per volume	Per cell
17. Transitional cell carcinoma	1·5	1·1
18. Transitional cell carcinoma	0·9	1·4
21. Renal cell carcinoma	2·0	1·9
22. Adenocarcinoma of prostate	1·4	2·0
23. Adenocarcinoma of stomach	0·7	0·6
24. Adenocarcinoma of colon	1·2	1·4
25. Adenocarcinoma of colon	0·7	0·7
29. Adenocarcinoma of bronchus	1·5	2·8
31. Leiomyosarcoma	5·0	3·0

Table 4. *Summation of nucleic acid and cellular measurements evaluating the significance of changes in human tumours in relation to homologous normal tissues*

	Increased	Slightly increased	Unchanged	Slightly decreased	Decreased	Total no. measured
Thymonucleic acid:						
per unit volume	14	8	1	3	0	26
per cell	12	10	0	4	0	26
Ribonucleic acid:						
per unit volume	4	2	7	3	0	16
per cell	4	3	7	2	0	16
Mean cell size	4	6	2	7	7	26
Relative volume of:						
cytoplasm	3	1	1	8	7	20
nucleus	7	4	1	5	3	20
nucleolus	0	0	4	1	5	10
chromatin	3	2	1	2	2	10
nuclear sap	5	0	0	1	4	10
Percentage of cell volume:						
cytoplasm	0	2	1	15	2	20
nucleus	10	8	0	2	0	20
nucleolus	2	3	0	1	4	10
chromatin	6	2	1	1	0	10
nuclear sap	4	0	0	4	2	10
Percentage of nuclear volume:						
nucleolus	1	1	2	2	4	10
chromatin	1	5	0	4	0	10
nuclear sap	1	3	0	4	2	10

for normal tissues as well as lesser degrees of change are totalled in the designated columns. The significance of the differences for thymonucleic acid per unit volume and per cell was determined by statistical means.

Differences greater than 50% from the normal for ribonucleic acid and nucleolar measurements or 25% for other cellular measurements were considered probably significant. The morphologic pattern of the tumours was characterized more by variability than by uniformity so that averages of mean values present only a partial picture. Slightly more than half of the tumour specimens had cells of smaller mean size than homologous normal tissues. This was true in only three of nine adenocarcinomas as compared with eleven of sixteen other carcinomas. The cells of hyperplastic epithelium were significantly larger than carcinoma cells in all six instances measured and smaller than carcinoma cells in nine of ten tumours, there being an average difference of 20% in each instance. Only four of twenty tumours had larger masses of cytoplasm than the corresponding normal tissues, and in eighteen the nuclei of tumours made up a larger percentage of the cell volume. The cells of eight of ten tumours had a larger percentage of chromatin. In two-thirds of the specimens measured, the nucleolus comprised a larger percentage of the volume of the tumour nuclei.

Despite the great variation in different tumours as well as within the same tumour, these findings suggest that many tumours are characterized by changes associated with decreased amounts of cytoplasm and relatively, but not necessarily absolute, increases in chromatin and other nuclear substances. The changes in cytoplasmic volume could represent a partial explanation of the increased amounts of thymonucleic acid per unit volume of tissue, but not of the increased amounts per nucleus. The computation of correlation coefficients showed that there is a tendency for the amounts of thymonucleic acid per unit volume to be greater in specimens composed of tissues having small cells and small masses of cytoplasm as well as cells with large percentages of nuclear and chromatin material. A careful comparison of results on individual specimens does not show a high correlation between the mean amounts of nuclear chromatin and thymonucleic acid. The explanation, which is not yet clear, could represent an increased concentration of thymonucleic acid on the nuclear chromatin of tumours, but confirmatory evidence is desired.

V. DISCUSSION OF NUCLEIC ACIDS IN TUMOURS

The work on nucleic acids and nucleoproteins in normal and neoplastic tissues has been reviewed in the recent literature (Beadle, 1945; Caspersson, 1946; Caspersson & Santesson, 1942; Davidson, 1945; Davidson & Waymouth, 1944*a*; Greenstein, 1944; and Stowell, 1945*d*). The role of these substances in the transfer of genic characteristics, in cellular division, in the synthesis of proteins and probably in the formation or action of certain enzymes will not be recapitulated. However, the evidence that disturbances in the nucleoproteins and nucleic acids may represent an important factor in neoplasia will be summarized.

Growth and tumour-inciting agents. That nucleic acids and their constituents have a stimulating action on growth has been shown by numerous investigators (for references see Davidson, 1943; Stowell, 1945*d*). Willis (1945) has pointed out that the structural features of tumours are incompatible with the view that they arise from a single minute focus at a single point of time and enlarge solely by proliferation. It is possible that a progressive neoplastic change could spread from one or more focal points by means of growth-stimulating, diffusible, active principles derived from nucleic acids.

Although the virus theory no longer has substantial support as the cause of all cancer, it is recognized that viruses are associated with the production of some tumours (Rous, 1946). In this connexion, it is of interest that all of the viruses which have been studied, in common with other living matter, contain one or both types of nucleic acid. Taylor, Beard, Sharp & Beard (1942) demonstrated that the virus which elicits Shope papilloma in rabbits contains desoxyribonucleic acid. The substance in the milk of mice that incites mammary carcinoma contains a ribonucleic acid complex. It is an interesting speculation whether the nucleic acids in such tumour-inciting agents could cause neoplasia by producing a qualitative or quantitative disturbance in the nucleoproteins of cells. Caspersson & Hydén (1945), in a cytochemical study of viruses, state that the simplest forms of viruses need ribonucleic acid for their reproduction while the higher forms need desoxyribonucleotides. Although such observations on viruses associated with tumours have not been made, those studied were parasites on the nucleoprotein system of the cell.

Mutation theory of etiology of cancer. One of the prevalent theories of an intracellular cause of cancer is the mutation theory. There is, however, only limited evidence of change in the nucleoproteins of the genes. It is of interest that ultra-violet light of similar wave-lengths may produce (1) a progressive depolymerization of sodium thymonucleate (Hollaender, Greenstein & Jenrette, 1941), (2) gene mutations, and (3) cancer of the skin. It is possible that the action of the ultra-violet light in producing cancer could be mediated through transmissible alterations in the nucleoproteins. Avery, MacLeod & McCarty (1944; McCarty & Avery, 1946) isolated a desoxyribonucleic acid capable of inducing specific transmissible changes in pneumococci, but similar substances have not yet been isolated for other types of cells. The presence of a comparable type of thymonucleic acid in *B. coli* has been reported by Boivin, Delaunay, Vendrely & Lehoult (1945).

Observations such as those of Sonneborn (1943) and Spiegelman (1945) that cytoplasmic substances may play an important role in the transfer of characteristics may be explained by self-duplicating cytoplasmic nucleic acids or related substances. Their possible relationship to viruses and cancer has elicited interesting speculation by Darlington (1944), Haddow (1944), Potter (1945), Thomas (1945) and Woods & du Buy (1945).

The interesting work of Strong (1945*a*, *b*) shows that the genetic transfer of such traits as carcinoma of the stomach and breast in mice may be effected by exposure to chemical carcinogenic agents as methylcholanthrene. These recent experiments have opened a fertile field for speculation as well as investigation which may eventually modify our ideas on the mutation theory of cancer as well as the role of various types of nucleic acid and nucleoproteins. An initial slight alteration in nucleic acids or nucleoproteins could, during a variable latent period, lead to the progressive changes in growth characteristic of malignant cells.

Cytologic evidence. On the basis of a cytologic analysis of 565 neoplasms of the human oesophagus, colon, rectum, cervix, uterus, breast, skin, larynx and lung, Koller (1943) presents interesting evidence of disturbances of nucleic acid metabolism in tumours. Biesele and his associates (Biesele, Poyner & Painter, 1942; Biesele & Cowdry, 1944; Biesele, 1944) have reported increased number and size of chromosomes in many tumour cells. The observations in tumour tissues of stickiness, non-disjunction, displacement and clumping of chromosomes, of polyploid cells with increased number and volume of chromosomes, of more frequent mitoses, of alterations in nucleoli, and of multinucleate and giant cells all represent cytologic evidence of abnormalities of nucleic acids in neoplastic cells. These findings are more frequent in anaplastic tumours.

Macrochemical investigations. A survey of the macrochemical work on nucleic acids in tumours shows that, although some reports are unsatisfactory and others contradictory, the results generally indicate that tumours contain more nucleoprotein phosphorus and nucleic acid than homologous normal tissues (for references see Caspersson & Santesson, 1942; Davidson & Waymouth, 1944*a*; Schneider, 1945; and Stowell, 1945*d*). The finding of greater amounts of nucleic phosphorus and smaller amounts of lipid phosphorus in malignant tumours than in normal tissues has been ascribed to the increased content of nuclear material in neoplasms (Stern & Willheim, 1943). Differences in the chemical analysis of the nucleic acids of tumours and normal tissues have not been substantiated by most workers. The possibility that the nucleoproteins of similar composition may have quite different configurations and actions has not been adequately investigated.

Davidson & Waymouth (1944*b*, *c*) found that in tumours the total nucleic acid concentration as measured by nucleoprotein phosphorus was slightly elevated and that the acid-soluble nucleotide concentration was decreased. The concentration of both ribonucleic and desoxyribonucleic acid was increased in tumours, although the ratio of ribonucleic acid phosphorus to desoxyribonucleic acid phosphorus was decreased.

Schneider (1945) determined the desoxyribonucleic acid and ribonucleic acid content of a transplantable lung tumour and of rat hepatomas, induced by feeding *p*-dimethylaminoazobenzene, with homologous normal tissues and compared his chemical observations on liver tumours with the work of other investigators. He found that the desoxyribonucleic acid content was much higher in both the lung and liver tumour. The ribonucleic acid content of the lung tumour was much greater and the liver tumour about the same as the normal tissue.

Carruthers & Suntzeff (1946) compared the extractible desoxyribonucleic acid in the epidermis of mice, before and after painting with methylcholanthrene, with a transplanted epidermoid carcinoma. Their results are in general agreement with comparable earlier histochemical photometric measurements made in the same laboratory (Stowell, 1942, 1946*a*).

Although macrochemical studies indicate increased amounts of nucleic acids in many tumours, some of the discrepancies may be explained by differences (1) in assay methods, (2) or in reference standards such as wet or dry weight, (3) the inclusion of varying amounts of necrotic tissue and extraneous cells without adequate histologic controls, and (4) the failure of the recognition of the importance of such factors as the volume of the cell, nucleus and cytoplasm in the interpretation of results.

Histochemistry and cytochemistry. The observations of Caspersson & Santesson (1942) represent the most significant cytochemical evidence of disturbances in nucleic acids and protein formation in tumours. From their observations with ultra-violet light of changes in ribonucleic acid content of nucleoli and cytoplasm, they conclude that there are fundamental differences between the malignant cell and the normally growing cell in regard to the development of the system of protein formation. In the malignant cell the endocellular inhibitory mechanisms which limit the activity of the normal protein-forming system have more or less ceased to function. The heterochromatic or nucleolar-associated portions of the nucleus play a special and essential role in the growth and carcinogenesis of the cancer cell.

Caspersson & Santesson classify tumour cells into two main groups. The 'A type' has nuclei of ordinary size with distinct, moderate-sized nucleoli and a high concentration of desoxyribonucleotides. The cytoplasmic volume is below average but contains high concentrations of ribonucleotides and proteins. The 'B type' of tumour cell has a large nucleus with low concentration of desoxyribonucleotides and has proteins rich in hexone bases. The nuclear membrane is irregularly thickened and the large nucleoli contain considerable quantities of ribonucleotides. The large amount of cytoplasm is poor in protein and almost free of nucleic acid. Between the two extreme types are all stages of transition.

The A type of cell, which occurs predominantly at the periphery of the groups of tumour cells where the nutritional supply is better, is evaluated as being in the process of very intensive growth and multiplication. The mechanism of division functions with great intensity without positive diminution in the nucleic acid in the interphase, a condition comparable to that seen only in certain extremely early embryonic stages. The high content of cytoplasmic nucleic acid indicates an intense protein formation, but the effective development of abundant cytoplasm is prevented by the short intermitotic phases.

In the B type of cells, which is situated in the central areas of tumours where the nutritional supply is less adequate, the prominent nucleoli and nuclear membrane system are seemingly geared for an intense production of ribonucleic acids and proteins but the mechanism is inhibited. Because of the lack of nutritional raw materials, the protein-formation system in the cancer cell functions inadequately. Transition cells between the two types give the appearance of normal cells stimulated to moderate growth.

Caspersson & Santesson suggest that the cause of the disturbance in the nucleolar associated chromatin lies in either the regulation of the activity of the heterochromatin or in the heterochromatin itself and believe that the available evidence favours the latter possibility. The suggested hypothesis, which would regard the changes in desoxyribonucleoproteins as of a secondary nature, is attractive and the accumulation of additional evidence will be awaited.

Critical problems. In evaluating the working hypothesis that disturbances of nucleic acids and nucleoproteins represent an intracellular cause of neoplasia, there are many questions to be answered by the relative paucity of facts. At the risk of engaging predominantly in speculation and theory rather than with facts, yet in order to ascertain the present relative extent of our knowledge and ignorance, let us examine in the succeeding paragraphs some of the questions that must eventually be answered.

Is there a characteristic type of disturbance always present as a specific property of neoplastic cells only, and what types of alterations are present in other types of normal and pathologic growth? The evidence on this question from macrochemical and histochemical studies is inadequate. Although there is ample evidence of quantitative disturbances of one or both types of nucleic acid in many tumours, it has not been demonstrated in certain instances and insufficient work has been done with suitable comparisons on other types of growth such as rapidly growing regenerating and embryonic tissue and benign tumours. It is of interest that of a total of thirty-four tumours compared by histochemical methods with normal tissues, none had a statistically significant decrease in the amount of thymonucleic acid per unit volume of tissue or per cell. Different portions of tumours may vary considerably in their nucleic acid content, and it is possible that more malignant portions would show significant changes not evident in other areas which may have been measured. All portions of a neoplasm need not necessarily show equally either malignant properties or the substances characterizing malignancy.

Caspersson & Santesson (1942), by careful cytochemical observations, found abnormalities in the amount of ribonucleotides and proteins in malignant tumours and concluded that they had a special role in carcinogenesis. They made comparable measurements on various normal and pathologic conditions of growth and found that the rapidly dividing tumour cells showed a similar picture to certain extremely early embryonic stages. The transition tumour cell, between the two extreme types, had the appearance of normal cells stimulated to moderate growth. Although they were able to characterize the changes more fully than other workers, their answer cannot yet be regarded as final without further confirmatory evidence.

Are there qualitative or functional alterations as well as quantitative changes in the nucleic acids and nucleoproteins of tumours? Although there is scant evidence available it would seem possible, if changes in nucleic acids and nucleoproteins are the primary intracellular cause of neoplasia, that complex qualitative or functional alterations could be as important, if not more so, than simple quantitative changes. However, further speculation should await additional facts.

Are the changes in nucleic acids a primary factor in carcinogenesis or only one of the numerous secondary results of other changes? If the changes are primary, by what mechanism are they elicited and how do they produce their effects? Can the changes in nucleic acid content be correlated with morphologic characteristics of the tissue or with differences in their distribution? Only the previously mentioned cytochemical work of Caspersson & Santesson (1942) on ribonucleic acid and proteins offers a partial answer to these questions. Although the histochemical work on thymonucleic acid showed a tendency for correlation of the amounts with the morphologic pattern of tumour cells, it was not established either that the higher values of the measurements can be entirely explained by larger amounts of nuclear chromatin or that the changes in the morphology of the tumour cells were caused by increased amounts of desoxyribonucleoproteins. More anaplastic tumours did not have consistently increased amounts of desoxyribonucleic acid.

What is the relative role of the different nucleic acids and protein components of nucleoproteins in the disturbance in tumours? Our knowledge of the nucleic acid component of nucleoproteins, although relatively incomplete, is much greater than our information on the protein components. This, of course, does not justify the assumption that because we do not understand the complexities of the proteins that they could not be of great, or perhaps greater, significance than the nucleic acids.

The suggested answers to these questions show that we have only begun to obtain significant information on the role of the nucleic acids in tumours. It is hoped that the results and ideas suggested will not be accepted as final conclusions, but rather as stimuli to obtaining, by a suitable variety of methods, additional, more significant evidence for the explanation of the observed disturbances in nucleic acid, nucleoproteins and enzymes in malignant neoplasms.

VI. SUMMARY AND CONCLUSIONS

The relative amounts of thymonucleic and ribonucleic acid per unit volume of tissue and per cell were compared in a series of thirty-one human tumours and homologous normal tissues. For many of the specimens, the relative mean volumes of the cell, cytoplasm, nucleus, chromatin and nucleoli were measured. As compared with homologous tissue, the amount of thymonucleic acid per unit volume of tissue in the thirty-one tumours was increased in twenty-nine (statistically significant in twenty-one) and the amount per cell was increased in twenty-one (statistically valid in fourteen). In no instance was the amount significantly decreased. Ribonucleic acid content was increased in about half of the instances measured. A majority of the tumours had cells of smaller mean size with less cytoplasm. Tumour cells, in most instances, had larger mean percentages of nuclear and chromatin material, but there was not a constant, direct relationship between the thymonucleic acid content and chromatin content or the degree of anaplasia of the tumours.

There is ample evidence from this histochemical study of human tumours and from the results published in the literature that many tumours have a disturbance in either or both their ribonucleic and desoxyribonucleic acids. In discussing the significance of the increased nucleic acid content of tumours, it is concluded that there is insufficient evidence to accept as final any of the interesting possibilities which have been suggested.

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EXPLANATION OF PLATE

Figs. 1, 2. Stratified squamous epithelium (fig. 1) and adjacent epidermoid carcinoma (fig. 2) from the larynx in specimen 14. Feulgen reaction; 7 μ section; × 620. The carcinoma contained 2·18 times as much thymonucleic acid per unit volume of tissue and 1·16 times as much per cell as the normal epithelium.

Figs. 3, 4. Normal gastric mucosa (fig. 3) and adjacent adenocarcinoma (fig. 4) from specimen 23. Feulgen reaction; × 620. The epithelium of the tumour contained 2·09 times as much thymonucleic acid per unit volume and 1·82 times as much per cell as the gastric epithelium. The supporting stroma was carefully excluded from the measurements.

NUCLEIC ACIDS IN THE CELL AND THE EMBRYO

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This paper deals mainly with work which has been done in Brussels University during the war and after the liberation of Belgium; this work has been mostly concerned with a study of nucleic acid distribution in various tissues and in developing embryos. Another fruitful line of investigation has been the isolation of nucleoproteins in native form, and the study of their physical and chemical properties, both in the living cell and *in vitro*. The aim of these investigations is to reach a better understanding of the part played by nucleic acids in cell physiology and morphogenesis. The results are still very incomplete, but it should be remembered that Brussels University was closed by the Nazis for almost three years, and that the research conditions in Belgium are still far from normal.

It is now a well-established fact that there are two main types of nucleic acids present in all sorts of cells: thymo- and ribonucleic acids. The coexistence of nucleic acids both in animal and vegetable cells is now agreed upon by all: nucleic acids of both types have been isolated in purified form from vegetable as well as animal cells (Feulgen, Behrens & Mahdihassan, 1937; Davidson & Waymouth, 1944*a*; Grégoire, 1942, etc.). Progress in the study of the nucleic acid distribution within the cell itself rests mainly on the improvement in methods for the cytochemical detection of these substances: in the cell itself thymonucleic acid can be demonstrated with the Feulgen test, while both sorts of nucleic acids strongly absorb ultraviolet light and can therefore be estimated by the delicate spectrophotometric method worked out by Caspersson (1940). Nuclease preparations, as I have shown (Brachet, 1942*b*), also prove valuable tools for the cytochemical detection of nucleic acids; if a slide is treated with active ribonuclease, ribonucleic acid is broken down and the structures which contained it lose their basophily entirely. Thymonucleodepolymerase, which affects only thymonucleic acid, is also often useful in checking whether a Feulgen-positive body really contains thymonucleic acid.

It should, however, be borne in mind that the chemical reactions underlying these different tests are not yet fully understood: the mechanism of the Feulgen reaction is still a matter for discussion; Stedman & Stedman (1943, 1944), Choudhury (1943) and Carr (1945) have expressed strong doubts about the specificity of the Feulgen reaction; they even go so far as to think that the Feulgen test does not show thymonucleic acid, but that this acid would dissolve during the acid hydrolysis and react with the fuchsin sulphurous reagent: the coloured product would stain an acidic protein, present in the chromosomes according to Stedman, the so-called chromosomin. Stedman thinks that thymonucleic acid is not present at all in the chromosomes, and he suggests that it might be a constituent of the nuclear sap and the spindle. Stedman's unorthodox views have been challenged by Barber & Callan (1944) and by Caspersson (1944). Recent experiments carried on in my own laboratory are difficult to reconcile with Stedman's opinions: for instance, a short treatment at room temperature of the slides with active thymonucleodepolymerase removes thymonucleic acid; the chromatin does not stain any more with basic dyes, nor does it give the Feulgen test. According to Stedman, chromatin owes its basophily to chromosomin, not to thymonucleic acid; it can hardly be so, unless the enzyme removes both thymonucleic acid and chromosomin at the same time, a hypothesis for which we have no evidence. Carr's objection that the Feulgen test is not a specific chemical reaction, because partially hydrolysed slides treated with SO_2 still react positively, is not satisfactory either: Carr believes that the hydrolysed thymonucleic acid should react with SO_2, but we found no evidence for such a reaction *in vitro*. On the other hand, the Feulgen reaction is entirely inhibited, both *in vitro* and in the case of slides, when the partially hydrolysed nucleic acid is treated with SO_3NaH for 10 min., in a non-acidic medium. Finally, it was found that pure native thymonucleic acid still gives an intensive Feulgen reaction after it has been submitted to fixation with Zenker fluid, embedded in agar-paraffin and cut in microtome sections; when suspensions of birds' erythrocytes or isolated nuclei are hydrolysed for 5 min. in N HCl at 60°, even without fixation, no thymonucleic acid can be found in the acid fluid (Dische's method). It is therefore obvious that, in contradiction to Stedman's assumption, thymonucleic acid is not dissolved during the hydrolysis preliminary to the Feulgen reaction, at least after histological fixation or when it is bound to proteins. All these observations are in favour of the classical opinion that the Feulgen test is specific for thymonucleic acid.

The specificity of the ultra-violet method has sometimes been questioned, as other substances than nucleic acids might show a strong absorption at the same wave-length; tautomeric shifts produced by the irradiation might eventually alter the absorption curve (Graff & Barth, 1938; Mirsky, 1943). As regards the nucleases, we know next to nothing about their action on nucleoproteins, and their effects have only been studied on purified nucleic acids which probably are not present as such in fixed cells; furthermore, there have been reports that ribonuclease has some proteolytic activity (Schultz, 1941; Cohen, 1945). Reliable results can therefore only be obtained if all available tests are applied and give concordant results; it is also imperative, whenever possible, to check cytochemical results with microchemical estimations of pentose- and thymo-nucleic acids. For instance, thymonucleodepolymerase has no effect on the basophily of some spermatozoa and red blood cells; it would be a complete mistake to conclude that these nuclei are devoid of thymonucleic acid, since they give a strong Feulgen test; microchemical estimations show that they are rich in the said acid: the correct conclusion is that thymonucleic acid is not attacked by thymonucleodepolymerase in these nuclei; this is an interesting fact in itself, which deserves further studies on the nature of the thymonucleic acid present in these cells and on the links uniting this acid to the proteins. The value of microchemical analytical methods must certainly not be forgotten, even if these techniques are incapable of the same refinement as the cytochemical ones.

Extensive use of the ribonuclease test quickly gave us results very similar to those reported, simultaneously and independently, by Caspersson (1941 *a*), Caspersson, Landström-Hydén & Aquilonius (1941) with the ultra-violet absorption technique: ribonucleic acid is a constituent of the nucleolus and the cytoplasm (more exactly the ergastoplasm), although it is highly probable that chromatin—especially heterochromatin—contains a small amount of ribonucleic acid besides very large quantities of thymonucleic acid. The ribonucleic acid content of various cells differs greatly, and this was put on a firm basis by microchemical estimation of their pentose content. It was soon found out that ribonucleic acid is present in large amounts in cells which produce great quantities of proteins only: growing oocytes, cells of actively dividing tissues, secretory cells of the exocrine pancreas or the gastric mucosa. It is also present in the Nissl's bodies of the neurones and the somewhat similar bodies described by Berg in liver cells; in the latter case, the ribonucleic acid content substantially decreases in fasting animals, a fact also observed by Davidson & Waymouth (1944 *a*, *b*) and by Kosterlitz (1944). Plasmatocytes are also rich in ribonucleic acid (Zylberszac, 1941; Van den Berghe, 1942); an interesting observation, since these cells are often supposed to be responsible for the secretion of plasmaglobulins. During blood-cell formation there is a steady decrease of the ribonucleic acid content. Proliferation of a virus (polyedric disease) in silkworms leads to a considerable growth of the infected cells, together with a marked synthesis of ribonucleic acid in the cytoplasm and the nucleoli (Gratia, Brachet & Jeener, 1945). Stimulation of hormone secretion in the hypophysis also leads to an increased concentration of ribonucleic acid in the cells, as shown by Desclin and by Herlant. On the other hand, organs which have a high physiological activity, but which do not synthesize large amounts of proteins (muscle, kidney, for instance) contain small amounts only of ribonucleic acid. Thus, the existence of topographic links between high ribonucleic acid content of the cell and protein synthesis was emphasized almost at the same time by Caspersson (1941*a*, *b*) and by myself (Brachet, 1942*b*).

This finding incited us to carry on work already begun earlier (Brachet, 1939) on the *metabolism* and *enzymatic content of the nucleus and the cytoplasm*: it has often been supposed that the nucleus is the main centre of protein synthesis in the cell, a view which is also favoured by Caspersson (1941 *a*). The material chosen was the germinal vesicle of growing amphibian oocytes, since these are obviously the site of a strongly anabolic metabolism. The results are far from complete, as only a few enzymes have been studied, and no general conclusion can yet be drawn. A few interesting facts, however, stand out: the respiratory activity of the isolated germinal vesicle is very low and enucleation induces a small drop only in metabolic activity. None of the enzymes so far studied (dipeptidase, polypeptidase, arginase, nucleases, phophatases) was found in larger amounts in the nucleus than in the cytoplasm (Brachet, 1942*a*). It is well worth pointing out that Duspiva (1942), using a very delicate and precise technique, found no correlation between the dipeptidase content of the nucleus and the onset of vitellus synthesis: such a correlation exists, however, in the case of the cytoplasm where dipeptidase increases markedly when the first yolk granules make their appearance. These results suggest that there is no evidence that the nucleus is the site of an especially active metabolism; cytoplasmic dipeptidase probably plays a part in yolk protein synthesis; if the nucleus controls such a synthesis, it works in a very delicate and still unknown way. The results obtained in the case of the germinal vesicle agree well with Holter's (1936, 1938) results on sea-urchin's eggs and on amoebae, and with Dounce's (1943) data on isolated liver nuclei: all the enzymes he studied were found in smaller amounts in the nuclei than in the whole organ, except for alkaline phosphatase. This enzyme is present in the oocyte's germinal vesicle also, but not in very great quantities. This apparent discrepancy is probably due to the fact that the germinal vesicle is abnormally rich in nuclear sap and poor in chromatin: it thus seems likely that alkaline phosphatase is to be found in large amounts in the chromosomes themselves. We know now that this conclusion is correct, since various authors (Willmer, 1942; Krugelis, 1944) found chromosomes to give an intense cytochemical test for alkaline phosphatase; a particularly elegant demonstration of the same fact has been given recently by Danielli & Catcheside (1945) in the case of the giant salivary gland chromosomes.

Why chromatin usually has a high alkaline phosphatase content is by no means clear yet; as pointed out by Moog (1943), who studied the developing chicken embryo, there is not always parallelism between phosphatase content of the nuclei and mitotic activity. It seems, according to Moog, that alkaline phosphatase is somehow connected with embryonic differentiation. Our own observations, which have been made on *Tubifex*, Axolotl and *Xenopus* eggs (Brachet, 1946), entirely support Moog's opinion: in all three species, cleaving eggs and gastrulae nuclei yield only a very faint alkaline phosphatase reaction, and there is no striking difference between chromosomes and chromatin of resting nuclei. The reaction gets stronger in the neural tube during neurulation; but it is only when tissue differentiation starts that the nuclei begin to give a really strong reaction. Of some interest also is a recent study we made on the intensity of the nuclear alkaline phosphatase reaction in various organs of the mouse: there is a striking parallelism between the intensity of the reaction in the nuclei and the speed of the turn-over of thymonucleic acid P, studied by v. Hevesy & Ottesen (1943, 1945): organs with a high turn-over in thymonucleic acid P give a strong reaction (intestinal mucosa, testis, spleen), while the reaction is very faint in the nuclei of the organs where the turn-over is low (muscle, kidney, brain, avian red-blood cells). Moreover, the intensity of the nuclear alkaline phosphatase reaction is increased in rats' liver after partial hepatectomy; so is also the thymonucleic acid P turn-over, as shown by Brues, Tracy & Cohn (1944).

These findings make it very likely that the nuclear alkaline phosphatase is responsible for the replacement of phosphorus in the thymonucleic acid molecule. The fact that, according to Danielli & Catcheside (1945), the different bands of the giant chromosomes give phosphatase reactions of unequal intensities might well mean that the speed of the P turn-over is different in the various genes.

Let us now proceed further and try to isolate the nucleic acids from the cells in their native state, in order to study their properties: recent work by Mirsky & Pollister (1943) in the U.S.A. and by Jeener (1944, 1946) in Brussels has shown that *thymonucleic acid* associated with the basic protein *histone*, can be extracted from the nuclei with concentrated saline solutions. These extracts are highly viscous, show a strong rigidity when mechanical disturbances are avoided and a strong negative streaming birefringence: these physical properties are due to the shape of the long, thread-like thymonucleohistone molecules. Thymonucleic acid and histone are rather firmly bound together, since both constituents of nucleohistone cannot be separated by prolonged electrophoresis. On dilution with water, thymonucleohistone precipitates as an elastic gel; Jeener's experiments have shown that this gel strongly retracts on the addition of traces of calcium ions: this fact might be of importance, since it is known that the nuclei are extremely rich in calcium (Scott, 1943; Williamson & Gulick, 1942; Jeener, 1946). Recent work carried on in my laboratory by Dr Errera (1946) has proved that X-rays greatly reduce the viscosity and the rigidity of thymonucleohistone, probably by inducing a depolymerization of the long molecules; this observation might sometime prove of interest in order to explain X-ray-induced breakage of the chromosomes. It is very interesting that thymonucleohistone isolated from previously irradiated birds' erythrocytes shows a much lower rigidity than that from the controls. This result makes it very likely that thymonucleohistone is also modified by X-rays in the living cells, the main change produced by irradiation being probably a depolymerization of thymonucleic acid. However, it should be borne in mind that there is so far little evidence that thymonucleohistone is already present in the form of a thread-like molecule in the intact nucleus. This caution is necessary since Jeener (1946) has found out that thymonucleohistone does not behave like a fibre molecule when it is placed in solutions of normal ionic strength; it is only when the salt concentration is markedly increased that the characteristic physical properties of thymonucleohistone long molecules make their appearance. Finally, it is worth mentioning that these properties disappear whenever either thymonucleic acid or histone is digested by a specific enzyme: they thus depend on both constituents of thymonucleohistone, as shown by Jeener (1946).

The peculiar physical properties of thymonucleohistone are strikingly similar with those of the so-called *structure proteins*: these substances have been extracted from various tissues by Szent-Györgyi (1940), using an alkaline salt solution containing a high percentage of urea as a solvent. The extracts show the high viscosity, thixotropy, and negative streaming birefringence so characteristic of thymonucleohistone; they also contain phosphorus, and Szent-Györgyi believes them to be cytoplasmic nucleoproteins responsible for the structure of cell cytoplasm. We were able to show, with Jeener (Brachet & Jeener, 1944), that Szent-Györgyi's extracts actually contain thymonucleic acid, a specifically nuclear constituent; the structure proteins, as well as thymonucleohistone, lose their physical properties when they are treated with thymonucleodepolymerase. Structure proteins are thus a complex mixture of cytoplasmic and nuclear constituents, but they owe their physical properties to thymonucleohistone. It seems likely that Bensley's (1938) 'plasmosin' is likewise contaminated with thymonucleohistone.

Let us now come to *ribonucleic acid*: working in Prof. V. Desreux's laboratory in Liége, my coworker Chantrenne (1946), succeeded in isolating from yeast a zymonucleic acid which was almost pure, according to solubility and electrophoresis analyses. This substance has a higher molecular weight than the classical tetranucleotide; its electro-titration curve has been studied, both prior to and after deamination. The results obtained do not support the cyclic formula proposed by Takahashi (1932) and Makino (1935); on the other hand, they constitute no proof of the classic Levene & Bass (1931) formula either. The action of purified ribonuclease on this nucleic acid has also been studied by Chantrenne: the reaction is a monomolecular one, with a high Q_{10} (5·4 between 26 and 36°). As already found by Allen & Eiler (1941), ribonuclease liberates one secondary phosphoric acid group for every 4 atoms of P. The electrotitration curves show, furthermore, that no amino group enters in the link broken by ribonuclease.

The physicochemical behaviour of *cytoplasmic ribonucleoproteins* and thymonucleohistone is very different: the ribonucleoproteins can be isolated with the ultra-centrifuge as minute, spherical particles when tissues are extracted with dilute phosphate solutions. These ultra-microscopical particles (microsomes) have been studied by Claude (1938, 1939, 1940, 1943) and Stern (1939, 1945) in America, and by Jeener, Chantrenne (1944) and myself (Brachet & Jeener, 1944; Jeener & Brachet, 1941, 1942) independently. The whole of the ribonucleic acid present in dilute phosphate extracts of various tissues is bound to the granules; nucleic acid is, however, set free from the granules as soon as they are treated by acids, alkalies, strong salt solutions, heat, etc. Only in the case of embryos and yeast cells did we find soluble ribonucleoproteins resisting prolonged ultra-centrifugation: maybe these soluble nucleoproteins are somehow linked with the intense growth activity of embryonic and yeast cells. The granules always contain, besides ribonucleic acid, important respiratory enzymes like peroxidase, succinic dehydrogenase and cytochrome oxidase, which are likewise strictly bound to the granules: they are no longer present in the supernatant fluid after ultra-centrifugation. The same holds true for a special sort of phosphatide, the so-called plasmalogen. Other enzymes are present both in the granules and in the supernatant fluid: they include acid and alkaline phosphatases, amylase, dipeptidase, cathepsin, trypsin, arginase, adenylic acid deaminase, ribonuclease. Yeast lacticodehydrase, catalase and carboxylase are present in the granules, but in much smaller amounts than in the supernatant fluid. Some of these hydrolases are rather firmly bound to the granules and cannot be removed from them by repeated washings: for instance, the washing fluids extract only traces of alkaline phosphatase from the granules. All of the hydrolases are also present in the supernatant fluid, but usually in much lower concentration than in the granules.

It is a rather important point that the various constituents of the granules move together when whole living cells are being ultra-centrifuged: when a piece of fresh amphibian liver is ultra-centrifuged, the granules are thrown down towards the centrifugal end of the cells and packed together in a basophilic moon crescent. This part of the cells yields positive cytochemical tests for all typical constituents of the microsomes: ribonucleic acid, —SH rich proteins, plasmalogene, peroxidase and indophenoloxidase are now all localized at the centrifugal end of the cells; the same reactions are negative in the clear, centripetal part of the cells. In the normal, non-centrifuged cell, the reactions are given by granules dispersed throughout the cytoplasm. It also seems that a large proportion of the cytoplasm's acidophilic proteins (histones?) is bound in the granules: the major part of these proteins is displaced by ultra-centrifugation of the whole cell, although some of it is present in the clear hyaloplasm. It may therefore be concluded that all the chemical constituents of the granules move together on ultra-centrifugation, in the living cell as well as in tissue extracts; this observation brings strong support to the view that the granules are pre-existing chemical and physical entities in the normal cell.

Part of their physiological importance and significance can be understood. thanks to experiments carried on by my coworker Chantrenne (1944): he compared the oxygen consumption of normal and ultra-centrifuged entire frog's livers and found that sedimentation *in vivo* of the granules decreases the oxygen uptake by 50%. This inhibition is practically completely due to the inactivation of the cyanide-sensitive system, i.e. the Warburg-Keilin system: the ultra-centrifuged liver is practically cyanide insensitive. This result is easy to understand: cytochrome oxidase, in the living cell also, is intimately bound to the granules; when these are closely packed in one part of the cell, their functional activity is reduced, probably because other members of the oxidation chain have no longer free access to the cytochrome oxidase. Close packing of the oxidase-bearing granules in a small part of the cell thus exerts the same effects as cyanide: in both cases cytochrome oxidase activity is inhibited, either by physical or chemical means. Similar results had been reported earlier by Huff & Boell (1936) in the cases of insects and *Ascaris* eggs: ultra-centrifugation, here also, decreases respiration and inhibits only the cyanide-sensitive part. These cases, which seemed puzzling at one time, can now easily be explained.

The results clearly demonstrate that the ribonucleoprotein granules are pre-existing structures in the living cell, where they exert important physiological functions. Another point of interest is the following: when granules are isolated from red blood cells, they are found to contain a small amount of haemoglobin which cannot be eliminated by repeated washings. In the same way, pancreatic granules contain insulin, while the hormone controlling the expansion of melanophores is present in hypophysis granules. These facts point towards the following hypothesis: ribonucleoprotein granules might well be the agents of protein synthesis in the cell, as they always contain the specific protein synthesized by the cells in minute amounts, but in a firmly bound way. Such an hypothesis is consistent with many facts known about protein synthesis: the granules usually contain the whole of the ribonucleic acid, a characteristic constituent of all cells undergoing protein synthesis; a whole set of proteolytic enzymes is to be found in them, and there are also respiratory enzymes catalysing energy-yielding reactions. This constitution of the granules is the expected one, if the views of the biochemists, who hold that protein synthesis is an endothermic process catalysed by proteolytic enzymes and linked with energy-yielding reaction, are correct.

A possible explanation for the presence of large amounts of ribonucleic acid in cells which actively synthesize proteins might be found, as suggested by Chantrenne, in the fact that nucleic acids easily precipitate proteins; they might, therefore, facilitate protein synthesis in removing the reaction products of the synthesis. Their function would be comparable with conditions prevailing in Bergmann & Niemann's (1937) experiments, where *in vitro* synthesis of a dipeptide by a protease only occurs if the solubility of the newly formed peptide is very low. Possibly, both types of mechanisms (linkage with energy-yielding reactions and removal of synthesis products by precipitation) occur simultaneously in the granules.

Work on ribonucleic acid metabolism has been carried on along yet another line by Jeener, Wiame and myself (Jeener & Brachet, 1943, 1944; Wiame, 1946*a*, *b*): it is a well-known fact, since Van Herwerden's early work, that basophily decreases in yeast cells, when they are cultivated in the absence of phosphates; addition of phosphate to these 'exhausted' cells induces a sudden and considerable (up to fifteen times) increase in basophily. The interpretation was that zymonucleic acid is used up in the phosphorus-free medium, while it is quickly synthesized as soon as phosphates are added. Work carried on with Jeener showed that these considerable changes in basophily concern only the ribonucleic acid fraction which is not sedimented by ultra-centrifugation (so-called 'free nucleic acid'); the granules, on the contrary, keep constant their composition whether phosphate is present or not. There is no relationship between basophily of the whole cells and their multiplication: but there is a reasonable parallelism between the amount of nucleic acid in the granules and the growth of the culture: it is thus probable that the granules multiply during cell division, without any marked changes in their nucleic acid content.

On the other hand, there is no relationship between the basophily of the supernatant fluid nucleoproteins and cell multiplication; the 'free' nucleic acid undergoes synthesis on the addition of phosphate only if carbohydrate metabolism is going on normally: nitrogen can be removed from the medium without any effect, but the presence of an oxidizable or fermentescible substrate is indispensable. If this substrate is alcohol, for instance, which can be oxidized, but not fermented, synthesis of 'free' nucleic acid occurs only in aerobiosis and is stopped by cyanide; in the presence of glucose, synthesis is possible in anaerobiosis or in the presence of cyanide, but it is blocked by monoiodacetate, chloropicrine or fluoride which inhibit fermentation. 'Free' nucleic acid synthesis is thus closely linked with energy-yielding reactions.

More recent work, carried on by Wiame (1946*a*, *b*), has helped considerably in clearing the situation: 'free' nucleic acid synthesis is not really a nucleic acid synthesis, but a phosphorylation of nucleic acid. Addition of phosphate does not lead to any significant increase in purine nitrogen or pentoses, but a phosphorylated compound, highly basophilic, is formed. This substance can be isolated in fairly pure condition: it contains purines, pentose and about 17% P. It is probably identical with a substance isolated from yeast, a few years ago, by MacFarlane (1936), and which is thought to be a combination of zymonucleic acid and metaphosphoric acid.

Let us now come to a different topic, nucleic acid metabolism in developing eggs: these are, of course, the site of an intense nuclear multiplication accompanied by a considerable synthesis of thymonucleic acid. The existence of such a synthesis has been demonstrated in many types of animal eggs, the first demonstration of the fact being brought up in 1933 in the case of the sea-urchin's egg (Brachet, 1933); still another proof can be found in the fact that there is no viscous, birefringent constituent in unfertilized eggs, while thymonucleohistone can easily be demonstrated by its physical properties in the embryo. Chemical estimations of thymonucleic acid agree perfectly well in this respect with cytochemical observations using the Feulgen test. It is, however, a well-known fact that in many animals, including the sea-urchin, there is no synthesis of nucleic acid phosphorus or purine bases in early development; while in other forms, like the chicken embryo, these nucleic acid constituents are synthesized during development. There are thus two categories in respect of thymonucleic acid synthesis: either *partial* synthesis where thymonucleic acid arises from some other nucleic acid (in the case of the sea-urchin's egg) or *total* synthesis when all constituents of thymonucleic acid are synthesized (chicken embryo). The precursor of thymonucleic acid in sea-urchin's eggs is, most probably, the ribonucleic acid present in cytoplasm: the pentose content of the egg decreases during early development proportionally to the thymonucleic acid synthesized. In the chicken, on the contrary, there is a synthesis of both types of nucleic acids. More recent cytochemical work shows that cytoplasmic basophily, due to ribonucleic acid, decreases during sea-urchin's eggs development; however, when the gut begins to differentiate, its cells show an increase of basophily. On the other hand, in the chicken embryo, there is a persistent increase in basophily during early development, followed by a decrease in later stages. But cells undergoing mitosis, in the chicken embryo, always show a lighter staining of the cytoplasm than the neighbouring ones. This is by no means a peculiarity of the chicken embryos: in all types of tissues there is a marked decrease of the cytoplasmic ribonucleic acid at metaphase; simultaneously, the thymonucleic acid content of the chromosomes increases markedly: their basophily, Feulgen reaction and, according to Caspersson, selective ultra-violet absorption are much stronger than in the resting chromatin. That the metaphase chromosomes are also richer in histones is made likely by Caspersson's (1941 *a*) data on the ultra-violet absorption curve and by Serra's (Serra & Queiroz-Lopes, 1943) report that the arginine cytochemical reaction is stronger in dividing chromosomes. Our own observations confirm these findings, in showing that the affinity for acidic dyes increases in the chromatin and decreases in the cytoplasm when dividing and resting cells are compared.

Utilization of ribonucleic acid at the time of thymonucleic acid synthesis is blocked by X-rays, as shown by Mitchell (1943), using the Caspersson technique: after X-raying the cells, an accumulation of ribonucleic acid was found in the cytoplasm. According to Stowell (1945), there is even a slight decrease in the thymonucleic acid content of irradiated tumour cells. It is therefore likely that in most (perhaps all) cells, there is a breakdown of ribonucleic acid during mitosis, and that this acid, just as in the sea-urchin's egg, is a precursor of thymonucleic acid. It is of interest to note that, according to recent work by P. Dustin, an opposite reaction occurs in pycnotic cells: when the nucleus degenerates, thymonucleic acid is apparently transformed into ribonucleic acid.

It is thus now clear that in all sorts of eggs, two antagonistic phenomena occur simultaneously (Brachet, 1945 *b*, 1946): a decrease in ribonucleic acid during mitosis and an increase of the same substance whenever there is a marked growth or any other sort of protein synthesis. The embryo's metabolism is therefore fundamentally the same, as regards nucleic acids, as in any adult organ. There is no reason to oppose two different egg types: in the chicken embryo, tremendous synthesis of new proteins occurs and ribonucleic acid synthesis is more important than its utilization: when growth slows down later on, the ribonucleic acid content of the cells decreases, as shown by Caspersson and Thorell. In the sea-urchin's egg, on the contrary, there is very little growth in early development and mostly cleavage into smaller cells: ribonucleic acid is used up, except in the case of the gut when it becomes functional and when, presumably, enzymes are secreted. The different behaviour of both egg types, for long a puzzling problem, can now be easily explained.

But there is another, perhaps still more important, embryological problem where nucleic acids are coming in the foreground: strong evidence that these substances play an important part in induction phenomena has recently been adduced. It is a well-known fact, since Fischer's (1935) early experiments, that nucleic acids, nucleoproteins and some nucleotides may induce (or rather evocate, according to Waddington's terminology) secondary neural plates when implanted in the blastocele. Although it is certain that sterol derivatives may act as powerful evocators, as demonstrated by Needham, Waddington and coworkers (Needham, Waddington & Needham, 1934; Waddington & Needham, 1935; Waddington, Needham, Nowinski & Lemberg, 1935; Waddington, Needham & Brachet, 1936; Waddington, 1938; Needham, 1942), there is little evidence yet that the sterols normally occurring in the gastrula are active. On the contrary, we know that organizers are as active as ever after thorough extraction of their lipids (Holtfreter, 1934; Barth, 1939). Barth obtained a protein from the gastrula which proved highly active; actually, this protein was found to be a ribonucleoprotein. Further indirect evidence for the view that nucleic acids may play a part in evocations is found in the fact that heating in the conditions which inactivate the evocator also destroys ribonucleic acid.

But there are, of course, more important arguments to support the view that nucleic acids may act as evocators: first of all, I noticed (Brachet, 1942*c*, 1945*a*, 1946) that when grafts of various tissues are studied with cytochemical methods 2 or 3 days after their implantation in gastrulae, the behaviour of the nuclei towards the Feulgen stain varies greatly: in some cases the chromatin does not stain any more, while in others it is still normally reacting. There is a close, although not perfect, parallelism between the cases where thymonucleic acid has been broken down and those where the ectoderm has responded with an evocation. Attack of ribonucleic acid by the host's nucleases is still faster and more complete than for thymonucleic acid. It was found that the gastrulae actually contain ribonuclease, and there is thus no doubt that nucleic acids in the implant are broken down by that enzyme.

Still more convincing results are obtained when nucleoproteins of widely different origins, animal or vegetal, are implanted into gastrulae: there is an almost perfect parallelism between the initial content in ribonucleic acid of the implants and the percentage of evocations: for instance, alcohol-killed, crystallized tobacco mosaic virus gave 70% positive results, while nucleoproteins extracted from the organizer gave only 37 and 10% evocations, depending on their content in ribonucleic acid. Here also ribonucleic acid in the implant is always broken down under the influence of the host's ribonuclease. If the implants are treated by purified ribonuclease previous to their grafting, their evocating ability falls almost to nothing: no evocation was obtained with killed tobacco mosaic virus which had lost its ribonucleic acid, and only very few positive cases (5%) were found in the case of the liver ribonucleoproteins isolated by ultracentrifugation and treated with ribonuclease. The untreated liver granules, in marked contrast, gave positive results in 69% of the cases. It is likely that the few positive results are due either to incomplete enzymatic removal of ribonucleic acid or cytolysis of neighbouring cells.

Recently, Holtfreter (1945) has shown that cytolysis is an important factor in inducing neural structures in epiblastic explants: the percentage of neural tubes is highest when numerous cells have undergone cytolysis. It is worth pointing out that ribonucleic acid has a particular behaviour in cytolysing cells: there is first a considerable increase of basophily, usually in a certain part of the cell. Whether this increase is due to a synthesis of ribonucleic acid from the yolk or just to a segregation of this acid in one part of the cell is not yet clear. Later on, when the cells start disintegrating, they entirely lose their basophily and become heavily pigmented. Cytolysing cells thus behave in a way similar to the implanted organs, which also lose their ribonucleic acid shortly after they have been grafted.

It is therefore very likely that the evocating activity of dead tissues and nucleoproteins is due to their ribonucleic acid or the breakdown products of this nucleic acid by the host's nucleases. The possibility that some active substance other than ribonucleic acid might be removed by ribonuclease together with that acid cannot, however, be completely excluded. The fact that nucleic acid-free lipo-proteins, serum proteins for instance (Brachet, 1946), are inactive as evocators make such a possibility rather improbable. It is, of course, a much more difficult, and still incomplete task, to establish whether these nucleic acids are also the inducing agents during normal induction in the gastrula. Some facts, however, suggest that ribonucleic acid may be part of the mechanisms which bring forward the induction of the nervous system in the living embryo.

Useful indications can be obtained in studying the topographical localization of ribonucleic acid during gastrulation and neurulation with cytochemical methods: these show (Brachet, 1945*a*) that ribonucleic acid is distributed in the egg, the morula and the blastula along a sharply decreasing animal-vegetal gradient. During gastrulation, the dorsal lip of the blastoporus contains more ribonucleic acid than the ventral marginal zone, a fact which has been confirmed by chemical estimation of the pentose content in the two regions. Towards the end of gastrulation, the ribonucleic acid localization is as follows in the invaginated archenteron roof: the concentration increases in the cephalocaudal direction, while in the upper-lying neuroblast an opposite, decreasing cephalocaudal gradient is to be found. Large amounts of ribonucleic acid are usually present at the interphase between archenteron roof and neuroblast, that is to say, exactly where inducing processes are at work. In the neurula stage, two gradients in ribonucleic acid are very conspicuous: a dorsoventral one in chordomesoblast and a cephalocaudal one in the nervous system. There is obviously a close relationship between ribonucleic and morphogenetic gradients, as well as between ribonucleic acid content and metabolic activity of the various parts of the embryo: this is not a surprising fact, since we know that ribonucleic acid and important respiratory enzymes are linked together in the same granules.

The occurrence, in large amounts, of ribonucleic acid in the membrane of the embryonic cells, especially in the neural plate, may have more than a casual interest: it has been pointed out by F. O. Schmitt (1941) that the deposition of a basic protein (histone) on a cell membrane charged with acidic groups might well induce the elongation of spherical cells; according to Schmitt, such a mechanism might conceivably be at work when the neural plate cells get their typical palisade shape.

It would obviously be going too far to assume from these observations that normal induction is due to ribonucleic acid or some of its derivatives; the mechanism of induction is probably very complex, and it may well involve many chemical reactions; but it seems highly probable that ribonucleic acid plays a part in these morphogenetic and chemical processes.

Experiments where ribonucleic acid metabolism was altered also provided interesting clues (Brachet, 1946): barbituric acid and benzimidazole, for instance, which probably interfere with nucleic acid synthesis in competing with uracil in the bacteria, strongly inhibit morphogenesis: the posterior half of the neurula is formed of undifferentiated gastrula tissues, while a more or less complete nervous system can be found in the cephalic region. When gastrulae are treated with acriflavine, which combines with nucleic acid and nucleotides, the nervous system in the neurulae is greatly reduced and sometimes absent, although a chorda and somites may differentiate. In such cases it seems that the archenteron roof has failed either to produce the inducing substance or that the presumptive neural plate is no more capable of reacting to the inducing stimulus. New experiments are needed to decide between these two alternatives. It is worth mentioning that both barbituric acid and acriflavine inhibit ribonucleic acid synthesis in the regions where morphogenesis is deficient. Another interesting point is that addition of nucleotides to acriflavine treated embryos often results in an improvement of morphogenesis. All these results suggest that ribonucleic acid or nucleotides play an active part in morphogenesis.

As regards nucleotides, recent experiments by Chantrenne and myself (Brachet & Chantrenne, 1946) showed that adenosinetriphosphoric acid undergoes a rapid synthesis during the differentiation of the isolated dorsal half of the gastrula; there is no such change in the ventral half. Furthermore, already in the early gastrula stage, there is a much larger amount of this important nucleotide in the dorsal half than in the ventral one. These findings are of some interest, since adenosinetriphosphoric acid is an active evocating agent, according to Fischer (1935); it is also well to remember that adenosinetriphosphoric acid plays a major part in glycolysis and constitutes an energy store in the cell. Perhaps this substance does play a similar role in inducing molecular changes in nervous system proteins as in myosin molecules during muscle contraction. Adenosinetriphosphatase, the enzyme which splits phosphorus from adenosinetriphosphoric acid, is also present in the gastrula, but its eventual localization has not yet been studied.

Discussion of the numerous hypotheses which come easily to mind would take us too far; it is, however, worth emphasizing that both during normal induction and evocation, there is a marked synthesis of ribonucleic acid in the reacting ectoderm; one of the consequences of induction is therefore a local synthesis of ribonucleic acid, which might well lead in its turn to the production of specific organ-building proteins. The main difference between induction and evocation lies in the fact that the normal organizer works along a microscopically visible gradient, while abnormal implants act only locally in a more anarchic way. Whether the chemical substance which induces nucleic acid synthesis in the ectoderm is a nucleotide or a sterol-like substance remains to be seen; it is interesting, as regards steroids, to remember that Graffi (1939, 1940) found a selective accumulation of carcinogenetic compounds precisely in the nucleoprotein granules of many different cells.

The fact that nucleic acids now come into the picture of induction is interesting, since it might help in clearing up the problem of genetic control in embryonic development. We know from Schultz and Caspersson's (1940) work that the chromosomic constitution of the nucleus influences the content in cytoplasmic ribonucleoproteins. Similar observations have been made on parthenogenetic and polyspermic frog embryos (Brachet, 1944): when a polyspermic larva is haploid on one side and diploid on the other, the haploid side often shows both poorer development and less basophilic cells. Still more interesting is the case of lethal hybrids between *R. fusca* ♀ × *R. esculenta* ♂: their development is put to a standstill at the onset of gastrulation, and ribonucleic acid synthesis is also stopped. If parts of such gastrulae are transplanted into a normal diploid host, they resume both development and ribonucleic acid synthesis.

Finally, a last point should be borne in mind: a large part of the ribonucleoproteins is present in the egg in the form of minute granules, comparable to viruses as regards both size and chemical constitution. A striking fact is—as such a distinguished embryologist as Dalcq (1941) has pointed out before these findings were made—the close resemblance between virus proliferation and the spreading in the embryo of the normally inducing substance; the multiplication of these ribonucleoprotein granules in the egg might thus well be of considerable importance for morphogenesis. These particles' importance would grow still more, if Haddow's (1944) and others' suggestion that these nucleoprotein granules might represent plasmagenes responsible for cytoplasmic differentiation should prove true.

Clearly, much work remains to be done along these and other lines; but it is an encouraging fact that progress is being made towards our ultimate goal: the establishment of closer links between such different sciences as cytology, embryology, biochemistry and genetics.

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THE ACTION OF ENZYMES ON CHROMOSOMES

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I. INTRODUCTION

The analysis of the chemical nature of the chromosome by means of enzyme digestion experiments which seek to identify specific substances and linkages by the action of specific enzymes was initiated by van Herwerden (1913) with nuclease and by Caspersson (1936) with trypsin and has been developed especially by Mazia & Jaeger (1939), Mazia (1941) and Frolova (1945). Trypsin completely digests and dissolves salivary chromosomes; but pepsin, though it drastically reduces the volume of the chromosome, does not destroy its continuity. These experiments are interpreted to mean that the chromosome has a continuous fibrous structure of a histone-like nature, digestible by trypsin but not by pepsin; in addition, there is a matrix protein, digestible by pepsin, which evidence of Caspersson (1940) suggests may be of globulin type.

Digestion of the thymonucleic acid part of the chromosome by nucleases (Mazia & Jaeger, Frolova) left the protein part intact, indicating that the thymonucleic acid played no part in maintaining the integral structure of the chromosome. The nucleases removed both the sugar (demonstrated by the loss of the Feulgen reaction) and the basic components (indicated by the loss of ultra-violet absorption) of nucleic acid from the chromosomes. Mazia (1941) found that inorganic phosphate inhibited the splitting of nucleic acid from the chromosomes by intestinal phosphatase, and concluded that the nuclease action is a phosphatase action, and that the nucleic acid was attached to the rest of the chromosome through its phosphoric acid. Further, intestinal phosphatase, supposedly freed from depolymerase by absorption of the latter on aluminium hydroxide, was still effective in removing nucleic acid from salivary chromosomes. This indicated that the nucleic acid was not present in the form of very high molecular weight units, but as moderately small polynucleotides.

We have attempted to extend this analysis by using purified thymonucleases, but find that doubt attaches to the specificity of some of the effects.

The disintegration of nucleic acid by enzymes is generally supposed to occur in three stages: (1) the breakdown of polynucleotide to simpler nucleotides by depolymerases, detectable by loss of viscosity; (2) the breakdown of nucleotides to phosphoric acid and nucleosides by nucleotidases (or phosphatases), detectable by the production of inorganic phosphate; and (3) the breakdown of nucleosides to carbohydrate and purine and pyrimidine bases by nucleosidases.

II. METHODS OF ENZYME PREPARATION

In assessing the importance of digestion with thymonuclease as a method for identifying thymonucleic acid in cells, it is plainly necessary to make some quantitative assessment of the enzyme preparations commonly used. Our experiments suggest considerable differences in behaviour of different preparations, which may well be due to enzymes accompanying the nuclease or depolymerase, but can be traced to other causes.

A chemical method was devised to give an estimate of the activity of every enzyme preparation used. In it, 1 c.c. of a 1 % solution of thymonucleic acid* was put with 1 c.c. of the enzyme solution in a small cellophane sac. Magnesium sulphate was added as activator. The sac was put in 10 c.c. of distilled water, to which a crystal of thymol was added, and was left overnight on the bench. Estimations of organic and inorganic phosphate were made on the dialysate. A small amount of sodium acetate was used as buffer, and the *p*H was about 6·8.

Three types of thymonuclease preparation were tested in this way, and at the same time their activity towards histological and cytological specimens was determined.

Rabbit serum, which Greenstein (1941) showed contained a fairly active thymo-depolymerase, was used as whole serum. Spleen thymonuclease was prepared by the method given by Fischer, Böttger & Lehmann-Echternacht (1941, 1943) for pancreas. The only modification made in this method was that the spleen was extracted with 10 % ammonium sulphate instead of water and dialysed at each stage against 1 % ammonium sulphate instead of water. Greater stability seemed to be secured in this way, perhaps because the ammonium sulphate inhibited the 'cathepsin' which is always present in these spleen preparations. Attempts to separate away the cathepsin have not been successful. Pancreas thymonuclease was prepared by the method of McCarty (1946). Until this method appeared, we used only spleen preparations, as we wished to avoid the risk of tryptic action. McCarty claims, and we confirm, that preparations made by his methods have no tryptic activity or but a mere trace.

Rabbit serum, though plainly able to reduce the viscosity of the thymo-nucleic acid solution, gave *no* phosphorus in the dialysate. The molecules produced were therefore not small enough to pass through the cellophane.

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* A highly polymerized sample very kindly provided by Dr K. Bailey and made by his modification of Hammarsten's method.

Spleen thymonuclease preparations caused the appearance of varying amounts of phosphorus in the dialysate, depending on the activity of the preparations. Roughly half of this phosphorus was present as organic and half as inorganic. These preparations plainly contain a phosphatase or nucleotidase as well as the nuclease.

McCarty's pancreas thymonuclease caused the appearance of large amounts of phosphorus in the dialysate, but none of it was present as inorganic phosphorus. This nuclease is a depolymerase, able to break the thymonucleic acid into sufficiently small particles to pass through the cellophane, without any obvious phosphatase activity.

In the spleen preparations with which most of this work was carried out, ribonuclease and the group of proteolytic enzymes jointly called cathepsin were always present and on many occasions were also estimated. No attempt was made to distinguish between the different catheptic enzymes but the activity was estimated at *p*H 3·8 by the digestion of denatured haemoglobin (Anson, 1939). Considerable activity was always found and may account for some of Mazia's results (1941) with unfixed glands at *p*H 5·0. There would undoubtedly have been considerable proteolytic digestion of his preparations at this *p*H, in addition to the nuclease effects for which he was looking.

III. EFFECTS ON CELL NUCLEI

The bean root tips used to show the effects of thymonuclease on cell nuclei in histological sections were fixed in 80% alcohol. Van Herwerden (1913), in an early paper on nucleases, showed that alcohol was the best fixative, many others making the nuclei entirely insensitive to thymonuclease digestion. After treatment with the enzyme solutions, the sections were stained with Unna's methyl green-pyronin stain. Serial sections were used as controls.

It was soon obvious that the spleen nuclease alone could not remove the blue staining material from the nucleus, but that, if preceded by exposure of the section to ribonuclease for 1 hr., it removed it completely. The ribonuclease removed the pink-staining substance from the cytoplasm and the nucleolus, but left the nuclei intact. The pink-staining substance is thought to be ribonucleic acid. The ribonuclease followed by the thymonuclease removed the blue-staining material from the nuclei as well.

McCarty's pancreas nuclease, on the other hand, removed the blue-staining material from the nucleus directly on its own, no ribonuclease being needed.

IV. EFFECTS ON SALIVARY CHROMOSOMES OF *DROSOPHILA*

Our experiments began with an attempt to remove the Feulgen-positive bands from salivary chromosomes by means of a preparation of spleen thymonuclease in the way that Mazia & Jaeger (1939) had done. The use of whole unfixed glands was early given up, because unfixed chromosomes became lysed and lost their structure, in preference for glands fixed in 45% acetic acid and smeared on cover-slips. The first spleen nuclease preparations, made according to the methods Mazia used (i.e. van Herwerden), behaved rather erratically, some judged highly active by chemical and viscosity estimations failing to remove the Feulgen-positive bands, while apparently feebly active ones stripped off the bands completely. It was thought possible that the erratic behaviour was consequent upon some impurity in the nuclease preparation.

Spleen preparations made by the method of Fischer *et al.* for pancreas nuclease were dialysed against water just before use to remove the ammonium sulphate. They were active as judged by capacity to depolymerize and to split thymonucleic acid, removed the Feulgen-positive bands from the salivary chromosomes, but not the similar material in bean root nuclei.

The thymonuclease prepared from pancreas by McCarty's method removed Feulgen-positive bands completely. This activity was shown whether abundant magnesium ions were added, or none. The activity was shown by solutions diluted to a point at which, judged by chemical methods, the concentration of enzyme was equal to that in an average spleen thymonuclease solution.

The spleen preparations are most active at *p*H 5, less so at *p*H 7 and inactive at *p*H 8. The pancreas preparations are most active at *p*H 7, and much less so at *p*H 5. This parallels their respective chemical activities in degrading thymonucleic acid and may provide evidence that they are different enzymes.

Various other nucleases have been tried. The depolymerase of dog serum and rabbit serum had no effect upon the bands. Spleen thymonuclease also is inactive in the presence of ammonium sulphate at 1% concentration. Pancreatic thymonuclease, when dilute, is also inactivated by ammonium sulphate. Quite different results from these are obtained if the dog serum, rabbit serum or spleen thymonuclease in ammonium sulphate solution are used after a pretreatment of the chromosomes by ribonuclease. Ribonuclease alone appeared to shrink the chromosomes slightly, as has been reported by Frolova (1945), but it caused no recognizable change in the bands which were as strongly Feulgen-positive after ribonuclease digestion as were untreated controls kept in distilled water or buffer. Following ribonuclease pretreatment, all the preparations, inactive on their own, completely removed the Feulgen-positive bands.

It was also found that a solution of egg albumin (an 8% solution of commercial egg white in 1% aqueous NaCl) which alone had no effect on the Feulgen-positive bands, following ribonuclease removed them completely. When recrystallized egg albumin was used, however, no removal of the bands was found. Possibly the effect depends upon an impurity present in the commercial preparation.

Trypsin was found, in agreement with Caspersson (1936), Mazia & Jaeger (1939) and Frolova (1945), to digest the chromosomes completely. Nineteen hours in a 1 : 5000 solution of commercial trypsin caused almost complete dissolution of the chromosomes, but the scraps left still showed well-marked Feulgen-positive bands. A stronger solution (1:500) caused no detectable digestion of the substance of the chromosome in 1 hr. at 20° C., but when followed by spleen thymonuclease in ammonium sulphate, after thorough washing of the chromosomes to remove the trypsin, the bands were completely removed.

Tests of the activity of our various enzyme preparations shows that the results obtained when they are used on fixed chromosomes is paralleled by the activity shown when they are used on fresh unfixed chromosomes. The only difference to be noted is that when unfixed chromosomes are used they rapidly (in 1–2 hrs. at 20° C.), become lysed losing their distinctive form. This lysis also occurs in distilled water at *p*H 7, but not in 1% ammonium sulphate or in weak acetate buffer at *p*H 5. The latter inhibition of lysis is not removed by subsequently putting the chromosomes into distilled water, so whatever causes the inhibition of lysis is irreversible.

V. DISCUSSION

Spleen and pancreatic thymonuclease remove the Feulgen-positive bands (presumed thymonucleic acid) from the chromosomes. The spleen preparations contain a depolymerase and a phosphatase (as well as cathepsin), the pancreas preparations a depolymerase, but no phosphatase. It seems difficult to believe that a depolymerase alone would be likely to loosen the nucleic acid from the protein, yet the McCarty enzyme does remove the blue staining material from suspensions of unfixed nuclei prepared from liver as well as the nucleic acid from nucleoprotein prepared by Mirsky's method. Preparations of pancreatic and spleen nucleases which split nucleic acid with equal activity, differ quantitatively in their behaviour towards nucleoprotein, the pancreatic enzyme being about twice as active as that of spleen in separating nucleic acid and protein. The interpretation of these differences in terms of the chemical links attacked cannot at present be attempted, but it is hoped that an analysis of the residues will be made.

The removal of the bands by rabbit serum, by egg albumin, or by spleen thymonuclease in ammonium sulphate solution, only after preliminary treatment with ribonuclease, may be non-specific, the nucleic acid being made labile or mobile by the ribonuclease (or equally, trypsin) treatment. It may be that the activity of the ribonuclease as a preparatory treatment for thymonuclease digestion depends upon a proteolytic activity. In the ordinary preparations used, ribonuclease has a proteolytic action towards the protein part of the nucleoproteins. Whether this is due to an impurity or to the ribonuclease itself is uncertain and is immaterial here, since it is known that such crystalline preparations as we have used (crystallized from ammonium sulphate solution by Künitz's method) are active in breaking down nucleoprotein.

In this connexion it is important to note that rabbit serum heated at 60° C. overnight with consequent destruction of the thymonuclease still removes the stainable material from bean root nuclei, after these have been pre-digested with ribonuclease. Further, human serum in which the thymonuclease content is known to be negligible (Greenstein & Jenrette, 1941) or at least extremely small [confirmed by one of us (B. Holmes)] removes the stainable material at the same rate as rabbit serum following ribonuclease. It is also known that protein solutions cause a considerable decrease in viscosity, i.e. presumably depolymerize thymonucleic acid. This may account for the non-specific effects.

This forms the basis of our objection to the conclusions drawn from previous work in which mixtures of nucleases with other unknown proteolytic enzymes, tryptic or catheptic according to the tissue from which the enzyme was prepared, were used. An enzyme such as cathepsin would be much more active at *p*H 5, at which Mazia (1941) worked, than at *p*H 7 and there is a danger that in such enzyme mixtures the effects may be non-specific in their character. One must therefore be cautious in concluding that a particular effect can be ascribed definitely to a particular enzyme.

The McCarty preparation of thymonuclease is the purest so far known and it should therefore be employed in testing any structures in biological material thought to be composed of thymonucleic acid. Equally it should be used to test any other substances extracted from the chromosomes and which are believed to account for the characteristic Feulgen stain (Stedman & Stedman, 1943). In favour of one constituent of the bands being thymonucleic acid is the fact that, like nucleoprotein, Feulgen stainability is removed from the bands by pancreas thymonuclease.

VI. SUMMARY

Thymonuclease (depolymerase), prepared from pancreas by McCarty's method, is the purest preparation so far known and readily removes nucleic acid from chromosomes. Thymonuclease (depolymerase and phosphatase) prepared from spleen removed thymonucleic acid from chromosomes. These effects are inhibited by 1% ammonium sulphate, but the inhibited preparations will remove nucleic acid after pretreatment with ribonuclease. Various protein solutions (rabbit serum, fresh or heated, and egg albumin) also removed the thymonucleic acid after ribonuclease pretreatment.

It is considered that the ribonuclease exercises a proteolytic effect in a preliminary loosening of the thymonucleic acid, the later removal by any protein being non-specific. The spleen and pancreatic thymonucleases differ from each other in their behaviour.

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THE FUNCTION OF DEOXYRIBOSE-NUCLEIC ACID IN THE CELL NUCLEUS

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Deoxyribose-nucleic acid, which will be referred to as nucleic acid in this communication, is recognized as a universal constituent of cell nuclei. Its presence may therefore be presumed to be essential to the existence of nuclei, and it may be inferred that it functions both as a structural and physiological component. In the exercise of these functions it doubtless acts in conjunction with other nuclear constituents. It is therefore unlikely that its functions can be satisfactorily and completely elucidated without a knowledge of the main nuclear components with which it is associated. We therefore propose first to discuss the chemistry of the cell nucleus in general and then to consider, in the light of this discussion, the possible functions of nucleic acid itself.

I. COMPOSITION OF CELL NUCLEI: CLASSICAL VIEW

While innumerable experiments have been carried out with cytological and microchemical techniques with the object of elucidating certain aspects of the chemical nature of the cell nucleus, relatively few direct chemical investigations have been made on isolated nuclei. This has been due to two causes: the difficulty of procuring, except in special cases, sufficient quantities of pure and uniform nuclei; and the apparently decisive results obtained in the early experiments on the composition of the heads of fish sperm and of the nuclei of avian erythrocytes, the two types of nuclei which can be isolated with relative ease. It is not the purpose of the present paper to attempt a complete review of the literature of the subject. A number of the early chemical investigations referred to above, some of outstanding merit, must, however, be considered because of the uniformity of their results and the consequent decisive influence which they have exerted on cytological theory.

The first and most important of these investigations is that of F. Miescher (1897), who, following a preliminary examination of nuclei from pus cells, undertook the chemical investigation of the heads of salmon spermatozoa. As is now recognized, this work was both brilliantly conceived and admirably executed, and it has probably never been surpassed in any subsequent investigation in this field. Miescher not only isolated for the first time the hitherto unknown compound nucleic acid, but he also demonstrated that it was an important component of the heads of salmon sperm, and that a similar, if not identical, substance was present in the sperm heads of other species as well as in the nuclei of certain somatic cells. At the same time Miescher succeeded in isolating from salmon sperm heads considerable quantities of an organic base, which he termed protamin. As judged from its properties, this base was of relatively low molecular weight; moreover, it responded neither to the xanthoproteic nor the Millon tests for proteins. Miescher, therefore, did not consider it to be a protein, and his opinion is still shared by some authors to-day. Nevertheless, it is now known that protamin is, like proteins, composed of amino-acids united by peptide linkings and, since the line of demarcation between a protein and a polypeptide cannot be sharply drawn, it appears, in our view, justifiable to consider protamin to be a simple protein.

Despite the brilliant outcome of his first investigation into the chemical nature of the salmon sperm head, it is apparent from his published correspondence that Miescher was not entirely satisfied with the result. His aim had been to separate the sperm head into its components as completely as if it were a mineral substance. That this object had not been achieved in his first publication is evident not only from its contents but also from the fact that Miescher continued to occupy himself with the subject almost from the time of the appearance of this paper until his death, some twenty years later, without the publication of any further results. Certain data, particularly of an analytical nature, were, however, available after his death. These were incorporated into a second paper and published under the editorship of Schmiedeberg. These historical details have been recalled because it was in this posthumous publication that the conclusion was reached that the heads of salmon sperm consisted to the extent of 96% of protamin nucleate, half of the remaining 4% being accounted for as inorganic material. This conclusion, frequently erroneously attributed to Miescher, has undoubtedly influenced subsequent workers in the interpretation of their less extensive experiments with other nuclei, and has thus tended to dominate our conception of the chemical nature of cell nuclei for decades. It must therefore be emphasized that, as Schmiedeberg explicitly states, the conclusion that the heads of salmon sperm consist to all intents and purposes entirely of protamin nucleate, while based on an interpretation of Miescher's analytical data, does not possess his authority but is a conclusion for which Schmiedeberg is entirely responsible. It was, moreover, only reached subsequent to Miescher's death. That Miescher himself would have dissented from it seems clear from his correspondence, for in the last period of his work on salmon sperm he had convinced himself that there was present in this material, in addition to nucleic acid and protamin, another substance which he termed karyogen. From Schmiedeberg's examination of this point it is probable that karyogen was never isolated in a pure state, and that some of the specimens of it which were analysed did, in fact, consist largely of protamin. However that may be, it is abundantly clear from Miescher's own writings that he was convinced from his qualitative and quantitative examination of salmon sperm heads that they contained material other than nucleic acid and protamin, and it is certain, therefore, that he would never have accepted Schmiedeberg's interpretation of his analyses.

Miescher's work on salmon sperm formed the model for many of the subsequent chemical investigations of cell nuclei. Kossel (1884) examined the nuclei from goose erythrocytes and found that, while they resembled the salmon sperm head in containing a basic protein in combination with nucleic acid, the protein was of a more complex nature than protamin. To emphasize this difference he introduced the name histone for it. In further work, extending over some years, on sperm from various species of fish, Kossel (1928) and his collaborators were able to demonstrate that all resembled salmon sperm in containing a basic protein. Some of these proteins were classified as protamins, others as histones. Meanwhile, Lilienfeld (1894) had isolated a nucleoprotein composed of a histone and nucleic acid from the thymus gland of the calf, which, on account of the very cellular nature of this organ, could be assumed to have originated from the nuclei. There was thus obtained evidence of the presence of a basic protein in fish sperm in general, and also in the nuclei of avian erythrocytes and in the thymocytes of the calf.

Apart, however, from Schmiedeberg's interpretation of Miescher's analytical data, the work outlined above contained no reference to the quantitative composition of cell nuclei. In 1897, however, a year after the publication of Miescher's posthumous paper, Mathews (1897), working in Kossel's laboratory, investigated the composition of herring sperm and reached the conclusion that the dry lipoid-free heads were composed of protein-free protamin nucleate. This conclusion was later confirmed by Steudel (1911–13), who further attempted to reinforce the certainty of his results by preparing a protamin nucleate from protamin isolated from herring sperm and nucleic acid obtained from the thymus gland of the calf. Steudel claimed that his synthetic material was practically identical with the sperm head in chemical composition, but he admitted that it differed from it in some of its physico-chemical properties such as optical rotation and viscosity. Nevertheless, some nine years later Steudel & Peiser (1922) stated that this work had demonstrated the identity of the synthetic and natural materials, while Ellinghaus (1927) obtained strong support for this conclusion with calorimetric measurements on the two products. In addition to the above investigations of the sperm heads of herring and salmon, an examination of that of the common white fish (*Coregonus albus*) was made by Lynch (1920). Using methods similar to those previously employed by Mathews, in whose laboratory the work was carried out, Lynch concluded that the sperm head of this species, like that of the salmon and herring, was 'essentially a chemical compound consisting of the union of nucleic acid and protamin in the proportion of four molecules to one'.

Another piece of work which extended to a different type of nucleus, the conclusions regarding the chemical nature of sperm heads had, meanwhile, been carried out by Ackermann (1904). Working in Kossel's laboratory, this author examined the nuclei from fowl's erythrocytes and isolated from them, as Kossel had previously done from goose erythrocytes, a histone. In addition, however, Ackermann concluded from his analytical data that the nucleus of the fowl erythrocyte was composed entirely of histone nucleate, although he did not so much as isolate any nucleic acid during his experiments. As Kossel (1928) states with regard to his results: 'He found that 100 grams of the dried nuclear substance contained 42·16 grams nucleic acid. From the total nitrogen of the nuclear substance the nitrogen left after deduction of the histone nitrogen corresponded with the assumption that 100 grams nuclear material contained 42·16 grams nucleic acid and 57·82 grams histone, and that no appreciable amount of any other nitrogen-containing substance was present.'

II. CYTOLOGICAL CONSEQUENCES OF CLASSICAL VIEW

If the results of the experiments outlined above be accepted, as they have been accepted almost universally, they carry with them two implications. In the first place, they suggest that all cell nuclei possess a similar simple chemical composition; in the second, they leave no doubt that one of the functions of nucleic acid is to participate in the formation of the chromosomes.

The first of these deductions has not, perhaps, been universally adopted. This has doubtless been due, in part, to the fact that, in his original work on the nuclei of pus cells, Miescher demonstrated that these nuclei contain a 'nuclein', as it was then termed, which differed from the nuclein (i.e. nucleic acid) subsequently isolated from salmon sperm in giving reactions for proteins. At the time Miescher's work was carried out, however, the existence of histones was unknown, and there is nothing to show that the protein reaction which he observed was not due to a histone. But if the view that nuclei in general are composed entirely of nucleohistones and nucleoprotamins has not been completely accepted, it has nevertheless been recently advocated by Mirsky & Pollister (1943) who claim to have isolated such nucleoproteins from various types of tissue in yields which would be expected only on this basis. They emphasize that the only proteins present in their nucleoproteins are protamins and histones, and they claim that the isolated nuclei from the thymus gland resemble fish sperm in yielding a nucleoprotein in an amount which represents nearly 90% of the material of the nucleus. Caspersson, as a result of his studies of the absorption of ultra-violet light by nuclei, holds a similar view of the chemical composition of the metaphase nucleus. On the other hand, his examination of the nucleus of the abnormal giant cell of the salivary glands of the larvae of certain Diptera, which he regards as an example of an interphase nucleus, leads him to the conclusion that the interphase nucleus, in general, contains proteins other than, and additional to, histones and protamins.

But, whatever the position may be with regard to the nuclei of secretory cells, if it be accepted that the heads of fish sperm possess the composition which has been claimed for them, it follows immediately that nucleic acid is a major component of the chromosomes, irrespective of what its ultimate function there may be. For, on current cytogenetical theory, the sperm heads must contain the paternal set of chromosomes and the only material present capable of conferring upon them the basophilic properties by which they are characterized cytologically is nucleic acid. Moreover, in view of the probability that the nucleic acids in the nuclei of various species possess identical compositions, it follows that protamin (or histone) must also be present in the chromosomes to confer upon them their function as the transmitters of inheritance. This view of the chemical nature of the chromosomes, deduced so simply from the studies of the chemistry of nuclei, is the one which is, of course, widely held. It has received much confirmation from cytological experiments and many cytological theories have been based upon it.

Such theories, however, give rise to a dilemma. They tacitly assume that the heads of fish sperm and nuclei in the metaphase of mitosis consist entirely of chromosomes. Some modern cytologists, in fact, use the term metaphase chromosomes in place of metaphase nucleus. Others are more explicit. Thus, in a recent review (Darlington, 1942), a description of chromosomes as compounds of histone and nucleic acid commences with the phrase: 'The nucleus consists of chromosomes which are composed of....' Another cytologist (Warmke, 1941), in tabulating the fundamental discoveries of cytology, describes the first one in these terms: 'The cell nucleus may resolve itself into microscopically discrete bodies, which we call chromosomes.' Similarly, a recent description (Caspersson & Santesson,

1942) of the process of mitosis includes the statement: 'During the middle period of cell division, the nuclear substance is entirely collected into the compact metaphase chromosomes which are composed of a dense, spiralized, gene-bearing, protein thread in which considerable amounts of nucleic acid are deposited.' Finally, two cytologists jointly state (Barber & Callan, 1944) that the heads of fish sperm contain no nuclear sap, a statement which can only mean that the heads are composed entirely of chromosomes. Yet it is well known that, at prophase of mitosis, the nucleus consists of chromosomes suspended or embedded in a medium known as the nuclear sap. Moreover, according to competent observers, the latter is transformed at metaphase into the spindle. Similarly, it is recognized by most cytologists that a sperm, on entering the cytoplasm of an egg, swells until it attains the size of the egg nucleus, when the chromosomes become visible. The nucleus must clearly contain the material which gives rise to these structures, namely, the nuclear sap and the spindle, although, on the basis of the results and theories outlined, none is available.

III. REINVESTIGATION OF COMPOSITION OF CELL NUCLEI

This contradiction can clearly be resolved only by a reinvestigation of the chemical nature of the nucleus. In an investigation of this kind now in progress we have already achieved results which demonstrate conclusively that the old view of the simple composition of the nucleus must be abandoned. This result not only gives scope for elucidating the composition of the nuclear sap but also obviously reopens the whole question of the chemical nature of the chromosomes themselves and hence of the function of nucleic acid in the nucleus.

The methods employed in this investigation, which have been outlined elsewhere (Stedman, 1944), do not differ in principle from those originally used by Miescher. The dry, lipoid-free nuclei, prepared according to Miescher's method in the case of sperm, or by an adaptation of Stoneburg's (1939) method in the case of all other tissue except erythrocytes, are extracted with dilute mineral acid to remove basic material, and the non-basic residue containing the nucleic acid then submitted to fractionation with dilute alkali. The scope of the investigation has been wider than those of earlier investigators and has included nuclei from fish sperm, bird and fish erythrocytes, liver cells from various species, thymocytes, lymphocytes, spleen cells, chick embryo cells and tumour cells of various types. The work is by no means yet complete, but the broad results as well as certain details which have a bearing on the chemical nature of the chromosomes and of the function of nucleic acid in the nucleus are available.

The results show that cell nuclei possess a qualitatively uniform composition, although considerable quantitative differences occur between different types. While all the nuclei examined do, in fact, contain nucleic acid and a basic protein, which may be either a histone or a protamin, these two components by no means account for the whole of the material present. There is always present in appreciable quantity another protein, which we have termed chromosomin (Stedman & Stedman, 1943), which is clearly distinguished by its properties from either histone or protamin. This result applies, moreover, to sperm heads obtained from the herring and the salmon as well as to nuclei from fowl erythrocytes and the thymocytes of the calf, a fact which is specially emphasized because these nuclei have hitherto been assumed to consist entirely of either histone or protamin nucleate. Particular care has been exercised in determining the composition of the heads of salmon sperm, partly because they were the first nuclei which were claimed to be composed entirely of protamin nucleate and partly because they are distinguished from many other nuclei, including the sperm heads of the cod and the herring, by a high content of nucleic acid, a fact which renders their examination more than usually difficult. The procedure used with one specimen of salmon sperm may therefore serve as an example of the methods employed. The sperm was obtained from the ripe testes of a salmon and was in a semi-fluid condition. It was worked up according to Miescher's procedure, when it yielded an aqueous suspension of what appeared on microscopical examination to be pure sperm heads without tails and uncontaminated with other material. Lipoids were removed by two extractions with cold alcohol and one with ether. The nucleic acid present was then assayed by a determination of the phosphorus content of the material dried to a constant weight over sulphuric acid in a high vacuum. This yielded a value of 4·80%. Taking the phosphorus content of nucleic acid as 9·90%, this gives a content of 48·5% for the nucleic acid in the dry, lipoid-free, sperm heads, a figure which obviously represents the maximum possible content of nucleic acid in the specimen examined. The bulk of the preparation was then exhaustively extracted with dilute hydrochloric acid and the protamin so removed converted into the sulphate and weighed as such. After correction for the combined sulphuric acid, the yield of protamin was found to constitute about 19% of the sperm head. It is possible that some protamin escaped extraction by this method. Its complete removal is, in fact, difficult in the case of nuclei, of which the salmon sperm head is the best example, which contain a large amount of nucleic acid relatively to its content of chromosomin. Nevertheless, the amount involved is exceedingly small and is insufficient to affect appreciably the above figure. From these results it is clear that the nucleic acid and protamin contents of the specimen of sperm heads examined account for less than 70% of the material present. It is, of course, impossible to assert that the whole of the remaining 30% consists of chromosomin. No doubt other substances are present in minute amount. Nevertheless, we believe that this residue is composed substantially of chromosomin on the following grounds. From an experiment in which 4 g. of the above preparation of sperm heads were employed, the residue left after removal of the protamin was fractionated repeatedly with dilute alkali. In this way it was separated into two fractions: a nucleic acid fraction contaminated with protein, presumably mainly chromosomin, and a protein fraction only slightly contaminated with nucleic acid. The latter protein is the substance which we have termed chromosomin. The yield in this experiment was 0·9 g. and thus represented more than 20% of the sperm heads. While this does not account quantitatively for the above-mentioned 30% of unknown material, the agreement appears to be sufficiently good, particularly in view of the difficulty of separating it quantitatively from nucleic acid (with which it has a great tendency to pass into solution in the presence of alkalis), to warrant the assumption that the 30% of unknown material consists substantially of chromosomin. In any case, these results demonstrate conclusively that the salmon sperm head is composed, not of protamin nucleate, but of three main components: nucleic acid, basic protein (protamin) and chromosomin. Similar results have been obtained with all the other nuclei examined, although, in general, their content of nucleic acid is lower, and of chromosomin higher, than in salmon sperm heads. Some of these results have been published elsewhere (Stedman, 1944). They will not be reproduced here because the method of calculating the results was somewhat different from that described above. They may therefore require slight revision when it is possible to redetermine them on exactly the same lines. From this work it is concluded that the above-mentioned substances constitute the major components of cell nuclei in general.

IV. NATURE AND DISTRIBUTION OF NUCLEAR COMPONENTS

Of these three components, nucleic acid alone has been subjected to detailed chemical examination. Numerous authors have been involved, yet there is apparently (Danielli, 1946) no general acceptance of the view that it is of invariable composition irrespective of the species from which it originates. It is beyond the scope of this communication to discuss this point in detail, but we believe that a review of the immense literature of the subject would yield substantial evidence in favour of this view, which is, moreover, strongly supported by our own experience with the compound isolated from nuclei of different types and derived from different species. In dealing with its function in the nucleus, the invariability of its composition will therefore be assumed.

In so far as they have been investigated, the basic proteins are known to vary in composition from species to species. This is evident from the fact that salmon and herring sperm contain a protamin whereas cod sperm contains a histone. Protamins and histones have, moreover, never been observed together in the same nucleus. One may therefore conclude that the two types of basic proteins exercise identical biological functions so that their differentiation is, from a physiological standpoint, superfluous. Nevertheless, the distinction is a useful one chemically, at any rate for the two best known protamins salmin and clupein, for whereas histones invariably contain aromatic amino-acids, none is present in these two protamins. Histones are usually stated to contain both tyrosine and tryptophan. According to Kossel, both thymus histone, which is the only histone of which any detailed investigation has been made, and that from cod sperm contain tryptophan, although the same author states that the slight tryptophan reaction given by the histone from the nuclei of goose erythrocytes is probably due to impurity. But in our examination of histones from a number of species we have consistently found that these proteins, while containing tyrosine, are completely devoid of tryptophan. This result applies, in particular, to the histones from the thymus gland and from cod sperm. We have, in fact, employed this absence of a tryptophan reaction as a criterion of the purity of preparations of histones, for, when contaminated with cytoplasmic proteins or with chromosomins, the reaction becomes positive.

These chemical properties of the basic proteins have been of great service to us in distinguishing between the various nuclear components. The tryptophan reaction serves to distinguish histones or protamins from the chromosomins, while, in the absence of tryptophan, the Millon reaction for tyrosine distinguishes between protamins and histones. This has enabled us to ascertain in a fairly simple manner the nature of the basic proteins in the nuclei of different cells from one particular species. For this purpose the salmon was selected because the sperm heads of this animal differ from the nuclei of most other species in containing a protamin as basic protein. In view of the probability, mentioned above, that protamins and histones exercise identical functions in cell nuclei, we were of the opinion that the difference between the protamin in salmon sperm and the histone in, for example, cod sperm was merely a species difference in the character of the basic proteins, and we consequently expected that the cell nuclei of the salmon would uniformly contain a protamin as basic protein. It is true that Miescher had failed to detect protamin in the nuclei from the unripe testes of the salmon so long as no spermatozoa were present, and had extracted, in its place, what he termed an 'albuminose', the nature of which is, however, unknown. But this experiment is difficult to repeat because spermatozoa appear at a very early stage in the development of the testes. Nevertheless, we have been able to determine the nature of the basic protein present in the sperm mother cells (presumably spermatogonia and spermatocytes) by indirect means. For this purpose we were fortunately able to secure the immature testes of a salmon weighing 20 lb. It is estimated from Miescher's data that the fully mature testes of a salmon of this size would weigh about 500 g. The actual weight was, however, only 39 g. Despite their immaturity, histological examination indicated the presence of a considerable proportion of spermatozoa together with the mother cells. The mixed nuclei obtained from these testes weighed in the dry and lipoid-free state 1·73 g. and yielded 18·5 % of their weight of the sulphates of the basic proteins. Qualitative examination of these sulphates demonstrated the presence in them of tyrosine and the absence of tryptophan, thus indicating the presence of a histone, while from the behaviour of the material in other respects it was evident, from past experience in handling protamin, that they also contained this protein. This conclusion from the qualitative examination was fully confirmed by a quantitative examination of the material, as can be seen from the following table:

Analyses of basic proteins from cell nuclei of the salmon

Source	N content of sulphate	Arginine-N (% of total N)
Sperm heads	22·6	87·7
Nuclei from unripe testes	20·6	66·5
Liver nuclei	15·75	25·3
Erythrocyte nuclei	15·6	19·0

The table not only gives the analyses carried out with the basic protein from the unripe testes, but also includes for comparison the results obtained with protamin sulphate isolated by the same method from the ripe testes. There are also included analyses of the basic proteins extracted from the nuclei of the liver cells and erythrocytes of the salmon. It is at once evident from the figures that the material obtained from the unripe testes contains less nitrogen and less arginine than protamin sulphate, which corresponds with the conclusion that it is a mixture of protamin and histone sulphates. Moreover, the analyses of the basic proteins from the nuclei of the somatic cells, coupled with the demonstration that they contained tyrosine but no tryptophan, offer incontrovertible proof that these proteins are not protamins but belong to the more complex class of histones. In this connexion it should be mentioned that all of the many histones from various species which we have examined form, under the conditions of preparation which we employ, sulphates containing approximately 16·0% of nitrogen. For example, that from the thymus gland of the calf contains 16·2%, a value which is identical with the one obtained with the histone from the nuclei of the liver cells of the ox. The entire character of the basic proteins from the somatic cells of the salmon is thus that of a histone, and there is little doubt that the occurrence of protamin is confined to the sperm, where it presumably plays the part of a substitute for the histone or histones which occur in the nuclei of the somatic cells and also of the sperm mother cells.

The histones from the liver cells and erythrocytes, while possessing sensibly the same nitrogen content, differ from one another in their content of arginine. We have no reason to doubt this result, which suggests that the nuclei contain different histones, but as the provisional analyses which we have obtained for the histones isolated from different tissues of the ox do not exhibit wide differences in composition of this kind, we prefer to withhold our judgement on this point until we have been able to procure sufficient material to confirm the analyses. There is, however, nothing inherently improbable in the occurrence of different histones in the nuclei of various cells of the salmon, for if, as is certainly the case, the sperm contains a protamin and no histone, while the somatic cells contain histones and no protamin, there is no reason why each type of somatic cell should not contain its own characteristic histone.

Chromosomin, the third main constituent of cell nuclei, has been so named, following normal practice, to indicate that it is the protein of the chromosomes, a point which will be discussed more fully later. It is present in every type of nucleus which we have examined for it and has been separated by the process outlined above in connexion with salmon sperm. As is the case with most proteins, it is difficult to characterize it in detail. It is, however, readily distinguished from histones and protamins by its lack of any marked basic properties. It further differs from these basic proteins, which do not contain tryptophan, in giving a strong reaction for this amino-acid. Like all other proteins it contains arginine and gives a strong reaction with the sensitive Sakaguchi reagent. It also contains tyrosine. These three amino-acids, namely, tryptophan, arginine and tyrosine, can be readily detected in proteins with the appropriate reagents, and, by this means, their presence has been demonstrated in the chromosomin separated from all the different types of nuclei which we have examined. These include the heads of salmon, herring and cod sperm, the nuclei from fowl and salmon erythrocytes, from liver cells, thymocytes, lymphocytes and myelocytes and from many tumour cells including both carcinomata and sarcomata. Nor has this survey been confined to one species. Similar results have been obtained, for example, with the nuclei from the liver cells of man, mouse, rabbit, ox, salmon, fowl and sheep. The tumours have been mainly of human origin, but two experimental tumours, one in the mouse and one in the rat, have also been investigated. In every case it has been possible to separate from the nuclei by a uniform procedure a protein possessing the properties which we regard as characteristic of chromosomin. It cannot, therefore, be doubted that chromosomin, like nucleic acid and histone (or protamin), is an essential component of cell nuclei in general. The detailed examination of its amino-acid composition has, however, been delayed by a number of factors. Apart from the difficulty or impossibility which obtained during the war, and which to a large extent still obtains, of procuring sufficient material and suitable apparatus for the work, is the added technical difficulty of completely removing from preparations of chromosomin of the last traces of nucleic acid. While, when one has gained experience with the lengthy manipulations involved, it is a relatively simple matter to isolate moderate quantities of chromosomin contaminated with a small percentage of nucleic acid, the removal of the last traces of the latter is a matter of considerable difficulty and is associated with great loss of material. This is no doubt due to the similarity between the general chemical characters of the two substances. In other words, as deduced by the fact that they dissolve in solutions of alkalis and are precipitated from such solutions by acids, they are both predominantly acidic substances. Nevertheless, in virtue of the basic groups which they contain, they must also be classed as amphoteric substances and, on this account, must be assumed to be capable of combining together chemically.

As finally isolated, chromosomin is practically insoluble in very dilute alkali although it swells to a marked degree in this solvent. For complete solution it is usually necessary to treat it with normal alkali, but even then many hours are required completely to dissolve it. From such solutions it is precipitated in granular form by acetic acid, when it can be redissolved with ease in dilute alkalis and again precipitated with acid. If, however, it is dried with alcohol and ether, it again assumes its insoluble character and requires treatment with normal alkali to bring it completely into solution.

As pointed out above, no detailed examination has yet been made of the amino-acid composition of chromosomin. Some provisional experiments have, however, been made with chromosomin isolated from cod sperm. Using a specimen which was slightly contaminated with nucleic acid (about 1 %), we have been able to demonstrate that its arginine content is somewhat greater than in many proteins although much smaller than in histones or protamins. The protein itself contained 15·7 % nitrogen and 20 % of this was due to arginine. This corresponds with a content of 9·8 % of arginine. As compared with this, the sulphate of the histone from cod sperm contains, according to our estimations, 13 % of arginine, which probably means a content of about 17 % for the free base. A similar estimation on the chromosomin from the Walker rat carcinoma gave nitrogen content for the protein of 15·9 %, 17 % of which was due to arginine, corresponding with an arginine content of 8·0 %. This result serves to show that the chromosomins from different species, while possessing the same general chemical and physical characters, and thus constituting a single group of proteins, are nevertheless individually distinct. In addition to these estimations of arginine, we have also isolated by standard methods from the hydrolysis product of 20 g. of the above-mentioned preparation of cod chromosomin two amino-acids, glutamic and aspartic acids, in pure condition. The former was obtained as its hydrochloride in a yield (crude) of 1·93 g. which, after twice recrystallizing, gave 1·21 g. of the pure salt. The yield of pure aspartic acid was 0·46 g.

V. FUNCTIONS OF NUCLEAR COMPONENTS

Having dealt with the main components of the cell nucleus, it is now possible to discuss their functions. For this purpose it is convenient to consider the nucleus as it appears during mitosis, since it is then composed of two sharply defined structures: the chromosomes and the nuclear sap. In taking this course, it is assumed that the composition of the dividing nucleus is not qualitatively different from that of the interphase or vegetative nucleus, although the latter would certainly contain the main components in different relative proportions. The term vegetative nucleus is here used to denote the nucleus of a fully differentiated cell which would not be expected normally to undergo further division.

The material of which the chromosomes is composed must clearly fulfil two requirements: it must be capable of accounting in a broad manner for the hereditary functions of the chromosomes, and it must possess, when in a condition corresponding to that of the fixed cell, basophilic properties. The first of these requirements can be satisfied only by one known type of compound, a protein. It follows that of the three main components of cell nuclei, either the basic protein or the chromosomin must be present in the chromosomes. A decision as to which is concerned presents little difficulty. The basic protein is, in fact, excluded on many grounds, the chief of which are based on a study of its nature and distribution. Both protamins and histones are regarded as simple proteins. Whether or no this description is accurate as regards histones is immaterial, for, in the spermatozoa of many fishes, which must contain the material of the chromosomes, protamin is the sole basic protein present and, certainly in the cases of the salmon and herring, it is of such a simple composition that it is inconceivable that it could play the complex role of forming the genes. Moreover, as we have shown above, while protamin constitutes the sole basic protein in the sperm heads of the salmon, it is absent from the nuclei of the somatic cells as well as from the sperm mother cells, where its place is taken by a more complex histone. If the principles of cytology are to be maintained, if the fundamental concept of the continuity of the chromosomes is not to be abandoned, it is clear from these results that neither histone nor protamin can enter in any essential manner into the composition of the chromosomes.

By a process of exclusion it thus becomes evident that chromosomin is a component, if not the sole component, of the chromosomes. Much further evidence of a more positive type can, however, be adduced in support of this conclusion. Chromosomin is a protein of complex nature such as would be expected of material forming the genes. As judged by the fact that it dissolves in alkalis, it is acidic rather than basic in character. Corresponding with, although perhaps not entirely because of, this it possesses, like the chromosomes of the fixed cell, basophilic properties, and thus satisfies the second of the above requirements postulated as essential for the material of which the chromosomes are composed. In particular, it is stained a deep blue colour when treated with haematoxylin (Mayer's haemalum), which it retains with great tenacity. Its response to this test for 'chromatin' seems of special significance when it is recalled that haematoxylin is employed almost universally in pathological laboratories throughout the world as a nuclear stain. Other nuclear stains, such as methylene blue, are less tenaciously held.

It has generally been assumed in the past that the basophilic properties of chromosomes are due to nucleic acid, and this substance is, in fact, frequently referred to as chromatin in the literature. Such a view has received apparent confirmation by the introduction of Feulgen's reagent into cytological practice. When cells are submitted to the technique of the nucleal stain, any chromosomes present are stained a characteristic purple or violet colour. The staining so produced is undoubtedly due to the presence of nucleic acid in the nucleus. A more precise interpretation has, however, generally been made of this effect, which has been considered to constitute proof that the nucleic acid of the nucleus is located in the chromosomes. This, in turn, has led to the assumption that the chromosomes are largely composed of this material. But in making this deduction one fact has been overlooked. The dye produced by the interaction of Feulgen's reagent and hydrolysed nucleic acid is completely soluble in water, as can be readily demonstrated in a test-tube with a pure specimen of nucleic acid. It follows from this indisputable fact that the dye can only be retained by the chromosomes because they consist of insoluble material which holds by adsorption or chemical combination the soluble dye produced in the reaction. This, of course, is precisely what happens with dyes in simple staining processes, except that the nucleus does not then contribute to the formation of the dye. The nucleal stain thus differs from simple stains merely in the fact that the effective dyestuff is generated inside the nucleus, which itself supplies the requisite nucleic acid, instead of being applied complete from outside. It is clear that no conclusion can be drawn from such results as to the location of nucleic acid in the nucleus. They throw no light whatsoever on this question. But they do demonstrate that the chromosomes are composed of material which combines with or adsorbs the soluble dye in question with very great avidity. This is a point of great importance, for this great avidity for 'developed nucleal stain', as the dye in question has been termed (Choudhuri, 1943), is shared by isolated chromosomin. If a solution of the dye is prepared in a test-tube by mixing hydrolysed nucleic acid with Feulgen's reagent and some chromosomin is stirred into the solution, it rapidly takes up the dye and assumes a colour similar to that of the chromosomes after staining with nucleal stain. The dye is, moreover, firmly held and withstands repeated washing with water. On the other hand, if the chromosomin is stirred for the same length of time with a solution of hydrolysed nucleic acid and is then washed several times with water, addition of Feulgen's reagent now fails to produce any appreciable colour. It is evident from this experiment that chromosomin does not possess the same avidity for hydrolysed nucleic acid (i.e. the active component) that it does for developed nucleal stain. It thus seems probable that the fuchsin moiety of the dye molecule contributes to a large extent to the great affinity between the latter and chromosomin. Other substances, in addition to chromosomin, are doubtless capable of adsorbing developed nucleal stain to some extent. For example, Gates (1942) states that if, in carrying out the nucleal reaction on plant cells, there is over-hydrolysis, the 'cell walls and other parts of the cell may show the violet coloration'. It can scarcely be claimed that plant-cell walls contain nucleic acid, but such results find an obvious explanation in the interpretation of the Feulgen reaction given above, and hence support this interpretation.

The above discussion, in our view, demonstrates quite conclusively that chromosomin is the essential component of the chromosomes, and that the basic protein contributes to the material of which the nuclear sap is composed. So far, however, the role of nucleic acid has not been considered.

While the discovery of chromosomin renders unnecessary the assumption, which has hitherto had almost universal currency, that nucleic acid is quantitatively the major component of the chromosomes, it does not necessarily exclude its presence from these structures. Nevertheless, our experiments provide cogent reasons for the belief that much of the nucleic acid of the nucleus is contained in the nuclear sap. It is, for example, scarcely possible to deny that histone and protamin, in virtue of their strong basic properties, are combined with nucleic acid in the nucleus. They combine together readily in the test-tube. Moreover, from the time Lilienfeld first isolated histone nucleate until the recent isolation of histone and protamin nucleates from different tissues by Mirsky and Pollister, they have been repeatedly extracted by gentle means from the thymus gland in combination with one another. If the necessity for this combination in the nucleus be accepted, it follows that such nucleic acid as is combined with the basic protein must, like the latter, be contained in the nuclear sap. In any case, unless nucleic acid, together with histone, constitutes the material of which the nuclear sap is composed, there seems from our experiments to be little else present in the nucleus in sufficient amount to serve this function. We therefore reach the conclusion that the two structures present in the dividing nucleus, namely, the chromosomes and the nuclear sap, are composed of chromosomin and histone nucleate respectively. The nucleic acid, partly or wholly combined with histone, must thus represent the matrix or ground substance of cell nuclei in general. In the nucleus of the vegetative cell the chromosomin must be assumed to be to a large extent dispersed in this ground substance or enclosed in nucleoli. In the dividing cell, however, the chromosomin separates or concentrates in the form of the chromosomes and the ground substance or nuclear sap changes at metaphase into the spindle. If this view of the function of nucleic acid be accepted, and from our experiments there seems to be no alternative to such a conclusion, it would appear that its presence in the nucleus is essential for spindle formation and hence for normal mitotic division. Its physico-chemical properties, it should be added, suit it admirably for this purpose. In the region of neutrality relatively dilute solutions of nucleic acid form a transparent or hyaline gel. The viscosity of its solutions is, moreover, extraordinarily sensitive to changes in chemical environment. Corresponding with this, the spindle at metaphase is known as a gel-like structure, the viscosity of which must diminish considerably during anaphase. If this change in viscosity occurs irregularly throughout the spindle, as doubtless it does, spindle movements would result which might well contribute to the movement of the chromosomes to the poles.

The role to be assigned to histone in this scheme of spindle formation is not at present clear, but it is postulated that it assists in the necessary orientation of the nucleic acid molecules, for which purpose an optimal amount would clearly be required. If this amount were exceeded, spindle formation and hence mitosis would be precluded. On the other hand, with suboptimal amounts of histone present the organization of the spindle would exhibit defects. This function of histone in its relationship to nucleic acid is suggested by our findings that the histone content of the nuclei of tumour and dividing cells appears to be low. This is illustrated by some figures for lymphocytes tabulated below:

Yield of histone sulphate from isolated nuclei

Source	Species	Yield (%)
Thymus gland	Ox	27
Thymus gland	Man	25
Blood (chronic lymphatic leukaemia)	Man	25
Blood (acute lymphatic leukaemia)	Man	7
Glands (same)	Man	9
Spleen (same)	Man	10
Liver (malignant lymphatic leukaemia)	Fowl	9

The lymphocytes from the case of chronic lymphatic leukaemia were found on histological examination to be normal small lymphocytes. Those from the acute case were mainly of a primitive type although a small proportion of normal cells was present. The malignant lymphocytes from the fowl liver showed numerous mitoses. These results clearly support the above views. As, however, they are incomplete as regards a knowledge of the nucleic acid content they will not now be discussed in more detail.

On the above views of the functions of nucleic acid and histone, the chromosomes would consist of threads of chromosomin embedded in the matrix material. The extensive surfaces of these threads would necessarily be in intimate contact with the nucleic acid of the matrix and it is probable that chemical combination would take place between them. Such combined nucleic acid could scarcely be regarded as a component of the chromosomes, but its effect would be to increase the acidic properties of the chromosomin and hence to enhance its basophily.

We do not, however, claim that our experiments give any proof that the chromosomes consist entirely of chromosomin uncombined, except superficially in the manner described above, with nucleic acid. But they do, in our opinion, show that the nucleic acid is not wholly concentrated in the chromosomes, and we therefore prefer the above conception of the chemical nature of these nuclear organs to any other which can be derived from the known chemical composition of cell nuclei. Moreover, at least one cytological experiment which supports this view is recorded in the literature, although it was designed to test our theory of the mechanism of the nucleal reaction and was apparently interpreted as refuting it. Soon after the first announcement (Stedman & Stedman, 1943) of our isolation of chromosomin from cell nuclei, Serra (1943) published a paper on the nature of the protein component of the chromosomes and on the mechanism of the Feulgen reaction. The final sentence of his paper reads: 'Il ne faut pas examiner d'autres arguments sur la relation entre la chromosomine et les colorations nucléaires puisque l'existence de la chromosomine est encore douteuse.' Yet in the experiments described in this paper Serra finds that after treatment of certain cells with a nuclease until the nucleic acid has been totally destroyed, the chromosomes stain in the usual way with iron haematoxylin. His actual words are: 'Les coupes se colorent très fortement après le mordençage et si l'on fait la différentiation on obtient de belles images de chromosomes etc., comme d'habitude.' If any interpretation can be placed on Serra's experiment it surely is that the chromosomes are so completely composed of chromosomin that the destruction of the nucleic acid in the nucleus has no influence on their structure.

It will, perhaps, add to the precision of our views on the chemical structure of the nucleus if we consider them in relation to some of the results which Caspersson has obtained in recent years by means of the ultra-violet microscope. We are at some disadvantage in discussing this point as few of Caspersson's publications have been available to us. From reviews of his work by others, and from one review of it by Caspersson & Santesson (1942), we understand that this author has submitted the giant chromosomes of the salivary glands of certain Diptera to extensive study and has interpreted his results on the assumption that they are typical of an interphase nucleus. Whether or no this assumption is valid, we are not in a position to decide. But the chief point to which we wish to draw attention is that certain of his results are not inconsistent with our own conclusions, although Caspersson interprets them quite differently. In particular, the finding of Caspersson that the interband sections of the euchromatin contains a 'higher' protein is quite consistent with our conclusion that the chromosomes are composed of chromosomin, which is, of course, a 'higher' protein if one uses as a criterion of such a protein a content of tryptophan and tyrosine. But Caspersson assumes that this higher protein is a synthetic product of the genes which accumulates in the interphase nucleus but breaks down and disappears before mitosis occurs. Consequently the metaphase chromosomes are, in conformity with his interpretation of his earlier work (Caspersson, 1936), composed of nucleic acid together with a relatively small amount of histone or protamin or of both these proteins. The dark bands of the salivary gland chromosomes are supposed to possess a composition similar to that of the metaphase chromosomes, although Caspersson admits that they may also contain some of the 'higher' protein.

In the light of our experiments a new interpretation must be placed on the presence of the higher protein in the interbands of the euchromatin. Far from being a synthetic product characteristic only of the interphase nucleus, the protein of the interbands evidently consists of part of the continuous thread or threads of chromosomin which run throughout the length of the chromosomes, as they do in the metaphase chromosomes, and thus represents an integral part of these structures. If the salivary gland chromosomes differed so fundamentally in composition from the metaphase chromosomes as Caspersson believes, it would be difficult to understand how they could be used so successfully by geneticists for locating the positions of the genes in the chromosomes. Such a procedure can only be possible if both types of chromosomes possess the same structure and hence the same chemical composition. The salivary gland chromosomes must therefore be considered identical in composition with the metaphase chromosomes and to differ from them only in the fact that they are present in the nucleus in extended form and have, perhaps, undergone greater growth without division. That the metaphase chromosomes are completely basophilic whereas only the bands of the salivary gland chromosomes possess this attribute doubtless depends on the greatly swollen condition of the chromosomin in the interbands of the latter, which prevents it from adsorbing sufficient dye, whether of the normal type or from the nucleal stain, to become visible. On the other hand, in the bands, which take up both types of stain, the chromosomin is in a highly condensed condition similar to that of the metaphase chromosomes and therefore becomes richly stained. Caspersson's view that the bands of the salivary gland chromosomes and the whole of the metaphase chromosomes contain protamin and histone is, of course, in direct contradiction with the conclusions which we have drawn from our own investigation.

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NUCLEIC ACID AND THE CHROMOSOMES

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I. INTRODUCTION

The last fifteen years (especially in the work of Caspersson and Brachet) have succeeded in showing a new principle at work in the cell. It is the reciprocal principle that, on the one hand, the nucleic acids are responsible for making the chromosomes, while, on the other hand, the chromosomes are responsible for making the nucleic acids. Reciprocal relationships of this kind are often found confusing or at least paradoxical, but their understanding is useful since they are characteristic of genetics. And this particular example lies at the root of biology.

The new situation created by our knowledge of the nucleic acids makes it possible, amongst other things, to rewrite cytology. It will have to be rewritten chemically in the next twenty years just as in the last twenty years it has been rewritten genetically. To undertake this task we, chemists and biologists, must be prepared to make great mistakes. We must be prepared to embrace and to reject hypotheses with appropriate recklessness. But, before starting on this course, we must agree about certain primary terms and notions.

II. THE FOUR ELEMENTS

In the first place, the chromosomes have been shown by recent experiments to consist of polypeptide fibres to which various elements are permanently attached. By their physiological and mechanical activity, these elements enable us to distinguish four types of structure in the chromosomes. The first is the *centromere* (Darlington, 1936), the organ of movement which secretes what Bernal (1940) has described as the negative tactoid of the spindle. The centromere has little or no nucleic acid attached to it. The second is the *euchromatin* which makes up the bulk of the chromosome and includes the active genes. These genes are separable from one another as large and visible *chromomeres* in the pachytene chromosomes at meiosis and in the polytene chromosomes of the Diptera. At these stages, Caspersson suggests, they happen to be engaged in protein production at the same time as they are charged with nucleic acid. The euchromatin passes through a nucleic acid cycle in mitosis: it is charged in the chromosomes when they are free, discharged from them when they are shut up in the resting nucleus.

The third element is the *heterochromatin*, so distinguished by Heitz (1935), which differs from the euchromatin in that it does not, as a rule, discharge its nucleic acid inside the resting nucleus but rather accumulates an excess. It is glutted and remains coiled up like a swollen piece of metaphase chromosome. It exists in large blocks which, at least in large chromosomes, do not include the centromere. Its chromomeres, which are visible only in the polytene chromosomes, are smaller, and its genes produce substances which are chemically smaller and genetically less specific and less obviously active (Caspersson, 1941; Darlington, 1942, 1944). Finally, the fourth element is the *nucleolar organizer* (McClintock, 1934), a granule which secretes or collects the nucleolus, a large body containing what seem to be heterochromatin products together with the cytoplasmic instead of the chromosomal nucleic acid. Both centromeres and nucleolar organizers are evidently compound genes, since they are capable of subdivision into workable parts.

Table 1. *The relation of heterochromatin to nucleoli*

(From Buchner, 1909; McClintock, 1934; Darlington, 1935, 1941; Heitz, 1935; Darlington & La Cour, 1940, 1941; Melland, 1942; La Cour, 1944; Koller, 1946*a*; *et al.*)

Nucleoli	Organizer	Heterochromatin	
		Present	Absent
Present	Not in heterochromatin	*Fritillaria pudica*, etc. *Paris japonica* ($8x$) *Allium cepa* vars. *Zea Mays* *Chironomus* spp.	*Fritillaria meleagris*, etc. *Paris quadrifolia* ($4x$) *Allium cepa* vars. *Hyacinthus orientalis* *Lilium regale* ♀ Orthoptera (No animals in heterozygous sex)
	In heterochromatin	*Drosophila* spp. Mammals (No plants)	—
	Absent	*Trillium* spp. *Paris polyphylla* ($2x$) *Rhoeo discolor* *Tulipa Schmidtii* *Pisum sativum*	*Fritillaria Elwesii* *Tradescantia bracteata* ($2x$) *T. virginiana* ($4x$) *Tulipa* spp.
Absent (No plants)	Absent	*Cricotopus* (Chironomidae)	—
	Present	♂ Orthoptera (testes)	

The relationship of these last two elements is less simple than it is sometimes thought to be. Many organisms lack either one or the other or both. When they lack both (as in *Tulipa*, Upcott & La Cour, 1936; *Fritillaria Elwesii*, Darlington, 1935), nucleoli are formed in numbers varying according to the tissue, at the ends of chromosomes in *Tulipa* (Woods, 1937), or at the centromeres in *Pisum* (Håkansson & Levan, 1942). When both are present the organizer may lie in the heterochromatin, as in *Drosophila* (Heitz, 1935) or man (Koller, 1946*c*) or outside it, as in *Chironomus* (Melland, 1942) or *Fritillaria pudica* (Darlington & La Cour, 1941). When there is no organizer or nucleolus at all as in *Cricotopus* there is always heterochromatin, and, where there is no heterochromatin, as in *Paris quadrifolia*, there is always a nucleolus. And finally, the appearance of heterochromatin is variable in animals. In the polytene chromosomes it is undercharged instead of overcharged. In female Orthoptera, at least in oogenesis, it is invisible, although the chromosomes which show it in the male are still present. A nucleolus appears instead (Buchner, 1909).

What is the meaning of these varying conditions? It might be argued that heterochromatin is always present, and always heterochromatic, but unrecognizable on account of extreme dispersion amongst the euchromatin. Comparison of related species with and without polyploidy and of the opposite sexes in Orthoptera suggests rather that the same genes which, in one species, sex or tissue, behave as heterochromatin, in another behave as euchromatin. On this view heterochromatin would depend on a special quality of certain genes which show *allocycly* in certain conditions (Darlington, 1941).

It may be that allocycly sometimes depends on the absence of a nucleolar organizer which can convert one nucleic acid into the other. For the nucleolus and the charged heterochromatin evidently provide alternative or supplementary sources of the nucleic acid used in the charging of the prophase chromosomes. It may be also that it depends on variations in nucleic acid supply which are greater in the course of animal than plant development. And it may be again that it depends on the lack of full employment of the heterochromatic genes by the euchromatin, for the genes, we must remember, employ one another. All these possibilities call for study.

The inertness of heterochromatin was formerly assumed for two reasons: the absence of major gene mutations, e.g. in the *Y*-chromosomes of *Drosophila*, and the absence of unbalance-effects arising from changes in number, e.g. of extra *B*-chromosomes in maize. Caspersson, however, infers that smaller proteins are produced by heterochromatin. Its chromomeres, which are visible with a reduced nucleic acid charge in polytene nuclei, are likewise smaller and less specific in their attractions. It seems, therefore, that the heterochromatin may be active but active in a way that is not recognizable by the methods of classical genetics. Recognition has now become possible by two new methods (Darlington, 1942, 1944).

The first method of recognition depends on the observation that small extra heterochromatic chromosomes maintain themselves in natural and artificial populations against the selective pressure of loss at meiosis. They reach an equilibrium characteristic of each population. They must therefore confer an advantage on organisms possessing them, whether in maize, in *Sorghum*, or in *Cimex lectularius* (Darlington, 1939 *b*), which compensates under natural or artificial selection for their own disadvantage. Again we may note that in *Tradescantia*, where there is no *visible* heterochromatin, similar fragments occur which are euchromatic (cf. Riley, 1944).

The second method of recognition depends on the demonstration that a heterochromatic segment (in the sex chromosomes of *Drosophila*) can exercise a special genetic action. This action is of the kind described as polygenic: its effects are slighter and less specific than those of the major genes, but they are of cardinal importance in all the processes of natural selection (Mather, 1942, 1944). The evidence from cell chemistry and chromosome morphology, from breeding experiments and from natural populations, therefore all concurs in showing the heterochromatin as differing from the euchromatin (where the two are distinguishable) in having smaller genes with smaller and less specific products.

In their mechanics as well as in their physiology the four elements of the chromosomes and their products are sharply distinguished. The centromeres and the euchromatic genes are mutually repelling structures; they do not stick together in healthy cells. The heterochromatin and the nucleoli are self-sticky; that is to say, pieces of heterochromatin fuse when close together in the nucleus (to form 'chromocentres'), and nucleoli likewise, but heterochromatin, or of course euchromatin, will not stick to a nucleolus. Indeed, a nucleolus will stick to a chromosome only through its own organizer. When the euchromatin is overcharged under abnormal conditions, due to temperature, irradiation or any other cause, it becomes sticky. Probably the clean, dry fibre so important for the regularity of mitosis depends for its character on the regular polymerization of desoxyribose nucleotides on the chromosome thread (Darlington, 1942; Davidson & Waymouth, 1944–5).

III. NUCLEIC ACID STARVATION

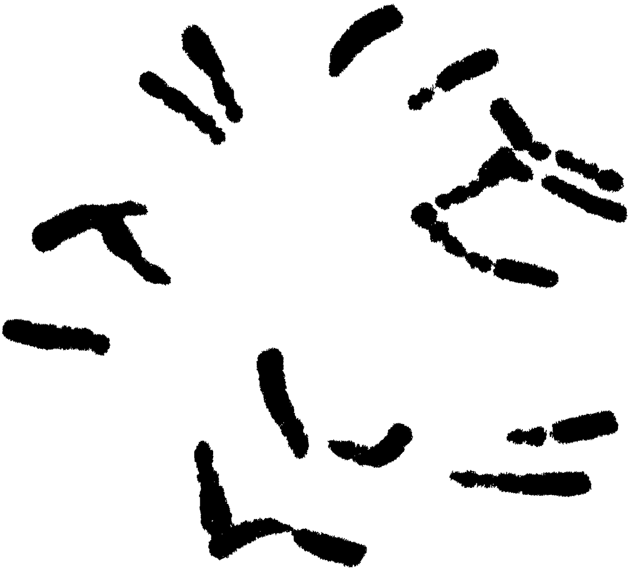
The chromosomes vary in their behaviour and appearance according to the nucleic acid supply available for their charging in the prophase of mitosis. This supply becomes reduced during the segmentation of the egg, and the chromosomes, apparently in consequence, become smaller (Brachet, 1944). Such variation in size likewise follows X-raying (Darlington & La Cour, 1945) and can also be genetically controlled (Darlington, 1937). In all these circumstances reduction in size might be due to a cut not in nucleic acid supply so much as in protein supply. It might entail a subdivision in a multiple gene string and a consequent economy in protein as well as in nucleotides. This would be a difficult assumption to make at present however.

A second source of differences in nucleic acid supply is differentiation. During the development of the blood in the bone-marrow of mammals the red precursors become differentiated from the white by having too much, as opposed to too little, cytoplasmic and chromosomal nucleotides (La Cour, 1944). Here the effects are of a new kind. With excess the chromosomes are heavily stained, over-spiralized and somewhat sticky. With deficiency they are under-stained and under-spiralized. Neither of these effects seriously changes the total chromosome size. Both are uniform for all parts of all chromosomes. With pernicious anaemia the normal differentiation is carried further. Indeed, it is carried too far, for both excess and deficiency in some degree paralyse the mitotic spindle. Deficiency usually leads to failure of the anaphase movement, and then the shortage goes so far as to leave the giant tetraploid resting nuclei so arising with their heterochromatin uncharged—its allocycly suppressed. Excess, on the other hand, leads to a multipolar spindle and the formation of many small and defective nuclei—whence the variety in size and shortened life of the red corpuscles with pernicious anaemia.

These effects of nucleic acid derangements on the spindles of the blood precursor cells seem to be derived from the primary effect of the nucleic acid supply in regulating the reproduction of the chromosomes. The chromosomes are too slow in the one case and too fast in the other for the cell components (or, should we say, protein component?) of mitosis to co-operate effectively. The ordinary differentiation of normal generative and starved vegetative nuclei in the pollen grain seems to be of the red-white type. The same is true of the differentiation of glutted nurse cells from eggs in animals, and from pollen mother cells in plants (Painter *et al.* 1942, 1943). Here again starvation prevents mitosis and glutting breaks it down, but the effect in each case is an adaptation profitable to the organism although suicidal to the aberrant cells.

Similar abnormalities depend on genetical variation. The action of particular genes in maize (Beadle, 1932) and of extra heterochromatic chromosomes in *Sorghum* (Darlington & Thomas, 1941) seem to upset mitosis by undue stimulation of the nucleic acid supply. In the *polymitotic* pollen grains of *Sorghum* the nuclei multiply as in a malignant tumour or the abnormal blood; but, the supply of nutrients being cut off, the encapsulated nuclei consume their own cytoplasm and die of mitosis. *Ne quid nimis.*

PLATE 1



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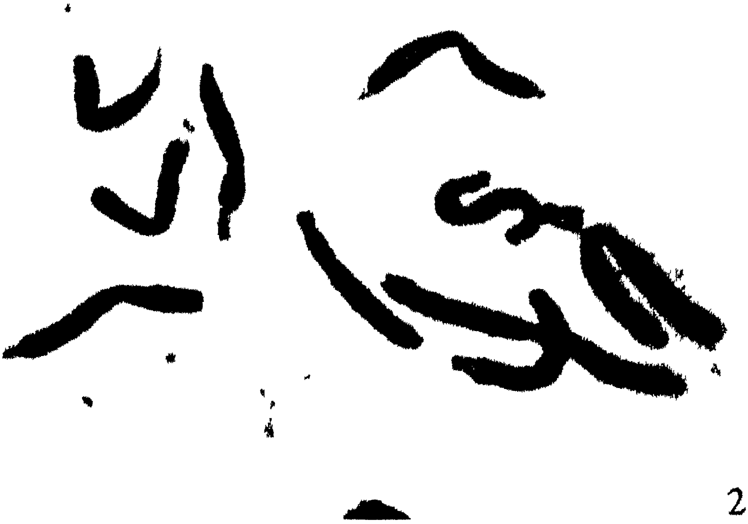

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For explanation see p. 269

The third source of upset in nucleic acid supply lies in a variety of conditions chiefly artificial which produce quite a different kind of result, one which explains much of what has gone before. When the prophase of a cell division takes place entirely at temperatures approaching freezing-point the heterochromatin (if there is any) fails to take up its full nucleic acid charge. It is therefore visibly different from the normal euchromatin at metaphase. This is true equally of plants and animals and of mitosis and meiosis. Metaphase starvability is a universal property of heterochromatin and is naturally a more reliable diagnostic than resting stage gluttability which must depend on a free supply. The cold-starvation effect shows in two degrees:

(*a*) *Half-starvation* produces a thinner chromosome segment, less deeply stained but spiralized to the usual length (Darlington & La Cour, 1938, 1940, 1941; Geitler, 1940). Occasionally division, especially of end-genes, is defective and the daughter chromosomes remain tied together as one continuous chromatid at anaphase. The obstetric action of nucleic acid in chromosome reproduction is thus established. This defect also explains the formation of chromatid bridges at mitosis and meiosis in organisms with defects of nucleic acid metabolism (Pl. 1).

(*b*) *Full-starvation* leads to failure of spiralization. The heterochromatic segment remains at metaphase as an uncoiled thread. This condition arises in two ways: (i) where there is no immediate supply of nucleic acid within the nucleus, as during the diffuse stage of the prophase of meiosis in the newt when the heterochromatin is uncharged and there is no nucleolus (Callan, 1942); (ii) where there is a reduced supply as in a polyploid species of *Paris* with very little heterochromatin (Darlington & La Cour, 1940).

Cold effects are presumably due to a shortage of chromosome nucleic acid within the nucleus. A cell shortage of nucleic acid arises under less precise conditions. It acts in both degrees. Half-starvation normally appears in the sex chromosomes before and at meiosis in mammals and Orthoptera. Full-starvation has been found only at meiosis in the X-chromosome of the hamster, *Cricetus*, again where the heterochromatic segment is very short. It arises from disease, lack of food, or old age. Adjoining and, what is more, synchronized cells may show full starvation and none at all. It is an all-or-nothing effect which has to be measured statistically. And where it approaches 100% failure of pairing sets in (Koller, 1946*a*).

Nucleic acid starvation is technically useful for many purposes. For our present theoretical purpose the observations confirm the view that the two types of nucleic acid are readily interchangeable. They also confirm the view that the chromosome type is both the dynamic agent of spiralization and also, as Astbury (1940) has suggested, the static template of reproduction, doing for the chromosome fibre with its sideways propagation what the cytoplasmic nucleic acid does for microsomes, plasmagenes and viruses (Claude, 1943). They show that the end-gene is particularly sensitive to error in this template when starved of nucleic acid. They show also that the heterochromatin may or may not suffer differentially from nucleic acid shortage. When it suffers, it suffers even more after being uncharged (and active) than after being charged (and inactive). They show further that the pairing of chromosomes is affected by extreme shortage of nucleic acid, even of chromosomes which do not show the shortage at metaphase. They show (negatively) that none of these abnormalities affect the centromere. And finally, they show that, while extremes lead to various errors, mitosis can take place within a wide range of nucleic acid supply. Or, to put it in another way: either protein or nucleic acid supply may limit cell division, but when the two are widely out of step starving or glutting with nucleic acid is the consequence. What these observations do not show is why nucleic acid starvation should have three different kinds of effect on the shape, size and differentiation of the chromosome. I can only surmise that starvation of different kinds is due to lack of different components of the nucleotides which are to be attached.

IV. CHROMOSOME BREAKAGE

Now we may see how irradiation acts on the cell system and on its various elements. X-rays have several different effects on cytoplasm and nucleus. These effects themselves interact, often before they can be recorded. Chromosome nucleic acid supply is first blocked and then flooded so that mitosis is first delayed and then hastened. The chromosomes entering mitosis immediately become sticky so that probably polymerization, as well as supply, is affected. In consequence cold-starvation may be removed where it has been induced, or imitated as a mere radiation-starvation where it has not been induced.

These cell effects are superimposed on other effects arising from the direct action of X-rays on the chromosomes. The chromosomes are broken in the resting nucleus. Their breakability is greatest at the end of the resting stage and in the most actively growing and dividing cells—those according to Caspersson's view with the least nucleic acid charge on the chromosomes. On the other hand, as a combination of starvation and X-ray experiments shows, it is virtually absent in the heterochromatin and in the prophase chromosomes, both of which are protected by their coat of nucleic acid.

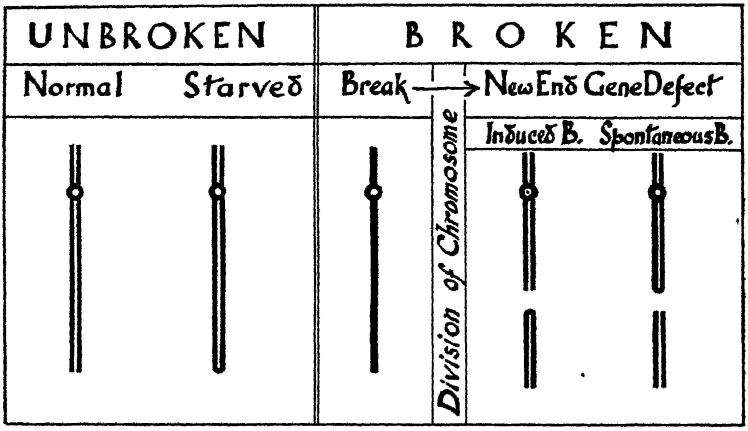
New ends of the chromosomes are exposed by breakage and these have two new capacities which help us to understand what makes an old end behave so quietly: the end-genes, or telomeres as Muller (1940) calls them, can reunite with one another in old or new combinations and they can also undergo non-division or 'sister reunion'. Neither of these capacities is shown by old ends except in the circumstances of nucleic acid starvation. Moreover, the broken end can lose the defective capacity for non-division during prophases, i.e. during nucleic acid attachment, except in the fastest mitoses. It can and does heal (McClintock, 1939). It looks as though non-division were due to the absence of a nucleic acid charge which is normally attached to true ends. True ends themselves can indeed sometimes unite with new broken ends after X-raying—after a nucleic acid upset. Failure of division of an end-gene therefore means failure to keep an old charge on an old end or to acquire a new charge on a new end, and the distinctive character of the telomere is due to an end-charge of nucleic acid (Darlington & La Cour, 1945).

These conclusions are borne out by certain remarkable statistical properties of rejoinability and non-division of the new ends. Both are related in frequency to breakability. Breakability and rejoinability are negatively correlated as they would be if both were dependent on the nucleic acid charge of the broken chromosomes. Thus a heavy charge seems to determine at once low breakage, and high reunion, frequencies, which is the reciprocal relationship to be expected if the nucleic acid is mechanically united with the chromosome. It also seems to determine a low frequency of non-division of the end-gene, which is in agreement with what we saw of the effects of starvation.

So far so good. But when we examine the occurrence of the non-division more closely we find that this seemingly trivial peculiarity tells us even more. The acentric side of a break suffers more than the centric. Hence acentric fragments always show the defect more frequently than those with a centromere (Text-fig. 1). Can it be that the centromere plays some part in organizing the nucleic acid attachment to the chromosome? Certain general considerations favour this view. Charging with nucleic acid at meiosis frequently begins near the centromere (Darlington, 1933) and pairing likewise (Darlington, 1940). Pairing, like reproduction, is in twos and is affected by nucleic acid abnormalities such as produce starved chromosomes, as we saw, or sticky chromosomes and persistent nucleoli (Klingstedt, 1939). The most conclusive evidence of the action of the centromere, however, comes from breakage arising in other ways than from irradiation.

Spontaneous breakage of chromosomes is found especially in pollen grain mitoses. Anywhere else under natural conditions it would be fatal.

It can occur either during the resting stage or at any later stage up to the separation of the chromatids at anaphase (Darlington & Upcott, 1941). When it occurs in the resting stage it simulates X-ray breakage. The imitation is perfect in every respect except for this defect in end-gene division. Spontaneous acentric fragments show, not less, but more non-division than their acentric sisters (Text-fig. 1). It seems as though spontaneous breakage in such a case is due to an error in the organization of the nucleic acid charge by the centromere, an error from which the breakage naturally relieves the acentric fragment.



Text-fig. 1. The relationship of nucleic acid starvation and breakage with the failure of an end-gene to divide as a permanent unstarved end-gene does. (Note: the difference in behaviour of centric and acentric fragments is relative.)

This speculation has been clarified by experiments on the effects of mustard gas on the chromosomes of *Tradescantia* at meiosis and afterwards (Koller, 1946*b*). These effects fall into a series according to the stage at which they arise. Resting stage effects cause changes like X-rays and spontaneous breakage. Later effects consist of breakage next to the centromere and correspond to the metaphase and anaphase types of spontaneous breakage. The whole series can be interpreted as due to the mustard gas acting specifically on the centromere and upsetting, either its organization of nucleic acid at the early stages, or its direct dynamic action at the later stages.

Spontaneous breakage can be reduced to rule. It can be adaptively controlled. For example, in *Ascaris megalocephala* the chromosomes in cells outside the germ line regularly break up into fragments during segmentation mitoses (White, 1936). Differentiation in nucleic acid supply between germinal and somatic cells might well be the basis of this system.

V. THE CENTROMERE

Evidently we can now no longer avoid the most difficult problem: what is this most miraculous organ, the centromere? When, some years ago, I gave the name centromere to the agent of chromosome movement I did so to emphasize its analogy with the centrosome and with the chromomere. I suggested even that the centrosomes could be centromeres escaped into the cytoplasm.

We can now take the argument a great deal further. The centromeres of unpaired chromosomes sometimes divide at the first instead of the second metaphase of meiosis. But sometimes (having failed to reproduce) they are unable to divide correctly by the regular lengthwise separation of identical halves. Instead they divide crosswise. This *misdivision* shows the centromere to be a compound gene whose division one way or another is forced upon it by orientation in the spindle. But misdivision has secondary effects. According to the proportions of the fragments new centromeres of different strengths are produced, some unequal to their task. Moreover, these centromeres are terminal. If they again misdivide a fragment of centromere may in fact escape into the cytoplasm. And if they do not misdivide they are liable to non-division just like other broken-end genes. When a terminal ordinary gene non-divides the result is, of course, a disastrous chromatid bridge at anaphase. When a terminal centrogene does the same, however, the result is the formation of a new chromosome with identical arms, an *isochromosome* (Darlington, 1939*a*).

These processes, observed first in plants, enable us to understand how it is that at meiosis in certain animals the centromeres, especially of unpaired chromosomes, may escape from them into the cytoplasm and take over the functions of centrosomes. This transformation, described by the Pollisters (1939, 1942), by all appearances arises from misdivision. The centrosome side of the case is therefore clear. The chromomere side remains.

On the face of it the centromere is a chromomere by nature starved or denuded at metaphase of the nucleic acid whose attachment to the rest of the chromosomes it is responsible for organizing during prophase. Even in the resting nucleus its nakedness in some sense is indicated by the mustard gas experiments. It remains a naked chromomere even at metaphase. Associated with this deprivation are the other properties that our experiments would lead us to expect. First, there is a delay in division. Secondly, when division takes place, there is a failure of attraction between its daughter halves. And thirdly, there is a failure of spiralization. But there is something more important that all these. The centromere appears at metaphase as an unstained constriction like that between chromomeres in the prophase of meiosis or in the salivary nuclei. And, like these chromomeres, the centromere continues to produce or, more strictly, to organize proteins, the proteins which we recognize as spindle fibres. By this process paired centromeres, or daughter centromeres, or (in the case of misdivision) half-centromeres, repel one another.

On the view that the development of the spindle depends on the protein production of centromeres and centrosomes its relationship with nucleic acid supply becomes clear. The activity of the centromere appears to be conditioned in two ways. On the one hand weak centromeres often appear in supernumerary chromosomes which arise by misdivision. They are also characteristic of univalents. Such centromeres act too little or at least too late. They do not make enough of the necessary proteins. On the other hand weak spindles, which are always hollow spindles, arise as we saw under certain conditions of nucleic acid starvation in blood and pollen, in myelocytes and microspores. Again, it seems, protein production is insufficient; but the insufficiency is due, not to a defect in the centromeres, but to a defect in the cell. At the other extreme an excess of nucleic acid in the cell goes with an excess of spindle development. Multipolar spindles result, it seems, from an over-stimulated protein production by the centromeres.

On this metabolic view of the centromere it might well be possible by a suitable derangement of the nucleic acid metabolism to repeat or imitate the change by which centromeres arose in evolution from ordinary chromomeres. This conversion has in fact been observed, once in maize (Rhoades & Vilkomerson, 1942) and twice in rye (Kattermann, 1939; Prakken & Müntzing, 1942). In rye it appears in inbred stocks selected for high vigour and fertility and it is associated with the excretion of nucleic acid from the metaphase chromosomes as well as non-division (or stickiness) of ordinary genes. In other words, it goes with a nucleic acid excess or disharmony following, perhaps, selection for high heterochromatin content.

The new centromeres are temporary. They appear at meiosis and disappear at the next mitosis. Thus they are associated not only with abnormal nucleic acid regulation of the whole organism but also with a type of cell division, meiosis, which, as we shall see, is inherently abnormal in this respect. They evidently arise after the division of the chromosomes at prophase for each is attached to a single chromatid. In this they are unique and mechanically significant. All true or permanent or old centromeres remain undivided until second metaphase and consequently hold pairs of chromatids together. Finally, the new centromeres are end genes. This is not surprising in view of the sensitivity of end genes to nucleic acid starvation. They are also most commonly the end genes of short arms, i.e. those nearest to the normal centromere. In some stocks they are confined to the end gene of a particular chromosome arm.

Naturally the new centromeres are weak and slow. In some plants they are so slow that they do not react with the spindle or with one another until anaphase. But in 'strong' lines they appear already at metaphase both in the first and the second divisions. And even at prophase they are strong enough to prevent complete terminalization of chiasmata. Their reactions are the usual ones at first metaphase. They lead to co-orientation of the usual types, linear, parallel and convergent, as well as independent. In univalents they are too slow and weak to secure, or to prevent, anaphase separation at the first or the second division. Their repulsions when discordant with respect to the old centromeres, do not break chromatids. At the first division, however, they show clear signs of misdivision in the appearance of a stretched end or satellite. This is to be expected since their single chromatids cannot divide correctly. At the second division also it must be noted that they co-orientate with the ordinary centromeres. This is also to be expected since it is found in those rare chromatid-centromeres which occur at the second metaphase, in the sex chromosomes of Heteroptera (Darlington, 1939*b*).

This transformation confirms what nucleic acid starvation, experimental breakage, misdivision and the origin of isochromosomes suggest, namely, that the centromere agrees in structure with other genes or chromomeres; that it is like them an agent of protein production but that it is one which continues its activity, by virtue of its nucleic acid starvation, during spindle stages, and that this starvation in the resting nucleus gives it special responsibilities in metabolism and special susceptibilities for breakage.

Thus the evolutionary order and the physiological relationship of chromomeres, centromeres and centrosomes are now becoming clear. The precise chemical changes involved, however, wait for an explanation upon our understanding of the nucleic acid relations of the three types of body.

VI. PAIRING AND MEIOSIS

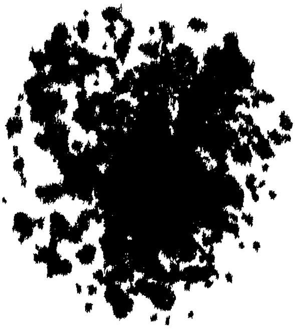
The control of nucleic acid supply by the centromere has its reciprocal aspect which is also of adaptive importance. The centromere with its low nucleic acid charge and lack of coiling divides after the rest of the chromosomes. In mitosis it divides at metaphase instead of during the resting stage. In meiosis it divides at second metaphase instead of at first prophase. In animals heterochromatic chromosomes, usually sex chromosomes, are often precocious, relative to the autosomes, in the division of the centromere at meiosis as well as in charging with nucleic acid. For example, the centromere of an *X* or *Y* divides at first, instead of at second, metaphase in

Hemiptera-Heteroptera (Darlington, 1939). The most remarkable abnormality, however, is produced by a gene complex known as 'sex ratio' which is widely diffused in species of *Drosophila.* It acts by way of gorging with nucleic acid the *X*- and *Y*-chromosomes which in ordinary flies bear the same charge as the autosomes. Both of these chromosomes are heterochromatic near the centromere, the *Y* more extensively than the *X*. The sex-ratio gene reveals their character at metaphase. Their pairing, which is by chiasma formation, fails. *X* divides twice instead of once. *Y* is paralysed by its glutted charge and is lost. All sperm, or nearly all, therefore contain *X* and beget nothing but females (Darlington & Dobzhansky, 1942). Thus centromere activity, nucleic acid metabolism and chromosome pairing and reproduction are again correlated and, for the first time, all at once; but here the correlation is natural and its effect manifestly adaptive, since it provides a parallel reproductive polymorphism in a whole group of species.

Diploid sperm mother cells of Orthoptera and other insects have a single *X*-chromosome. Aberrant tetraploid cells have two *X*'s. But being heterochromatic, overcharged and sticky they fail to pair (Makino, 1939; *et al.*). Perhaps it is not merely lack of nucleic acid but lack of clean polymerized nucleic acid which prevents pairing. On the other hand, the sticky heterochromatin seems to be responsible for the false or *secondary pairing.* Homologous bivalents at meiosis in polyploid plants lie too close together and thereby enable us to diagnose polyploidy. The attraction of blocks of heterochromatin for one another is also probably responsible for a preferential or *companionate segregation* which is seen in *Drosophila,* maize, *Neurospora* and *Cimex.* By this system pairs of genes, associated with heterochromatin, in different pairs of chromosomes pass to the same pole more often than they should, thereby limiting the randomness of Mendelian segregation (Thomas & Revell, 1946).

The association of both pairing and reproduction with nucleic acid supply makes it worth while to re-examine the view that pairing at meiosis is a substitute for reproduction, and that it arises when the chromosomes enter prophase before they have reproduced (Darlington, 1940). The entry of the chromosomes into prophase we now see as due to the deposition of nucleic acid on them. If this happens before the protein supply is ready for their multiplication, pairing should follow. The only known case of meiotic conditions replacing mitosis is in the pollen grains of a polymitotic maize (Beadle, 1931). Presumably the protein supply of the polymitotic *Sorghum* is not available in maize. The chromosomes are pushed into division before they have divided. Consequently they become over-spiralized as in an ordinary meiosis; and sometimes, although haploid, they even pair in some segments and cross-over. Precocity is a sufficient prime mover of meiosis.

PLATE 2



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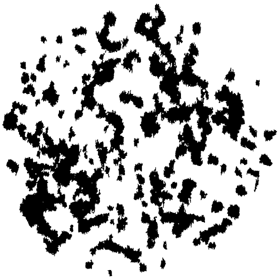

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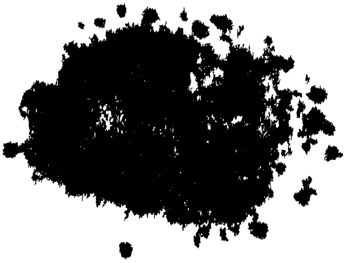

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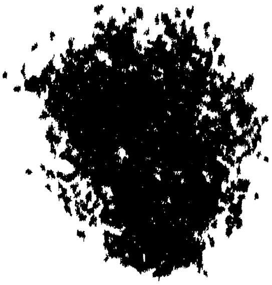

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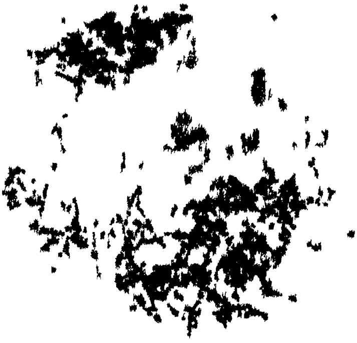

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PLATE 3

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The chromomere plays an important part in operating this precocity during the opening phase of meiosis. Caspersson (1941) has suggested that the chromomere is a unit of production charged with nucleic acid and separated from its fellows by its protein products. Pachytene at meiosis and polytene in the salivary glands are both distinguished from prophase of an ordinary mitosis by the appearance of these chromomeres, in other words by a premature deposit of nucleic acid. The mechanism of the deposit has been discovered in two steps. The first was by Painter (1943) who showed that the cytoplasm was flooded with ribose nucleic acid before pairing begins. The second is shown here (Pl. 2, figs. 3–8). There is an accumulation of masses of nucleic acid evenly distributed through the nucleus, and not obviously attached to the chromosomes, just before the beginning of prophase. These temporary dumps are even more striking in the embryo-sac than in the pollen mother cell of *Fritillaria*, and they occur equally in species with and without heterochromatin. They are distributed immediately afterwards to the chromosomes which thereupon begin to pair while their chromomeres are being separated from one another (Darlington & La Cour, 1946).

In animals the same flooding with nucleic acid is achieved in different ways, different even in the two sexes of the same species. In male Orthoptera the *X*-chromosome and one or two autosomes are glutted with nucleic acid in the early prophase of meiosis. In the female, on the other hand, the two *X*'s are entirely indistinguishable from the rest, and a nucleolus takes the place of the conditional heterochromatin as the nucleic acid store (Buchner, 1909; Bauer, 1933). The standard properties of meiosis which are so extraordinarily uniform morphologically (apart from the heterozygous sex in some animals) are no doubt capable of being achieved by many different chemical devices. But nucleic acid *priming* seems to provide the necessary condition of precocity, that is, of driving the nucleic acid cycle ahead of the centromere and protein cycle and thereby leading to two mitoses with only one division of the chromosomes.

Returning now to pairing we see that La Cour's observations on the development of chromomeres and Thomas's on the origin of secondary pairing now make it possible to put chromosome pairing into four categories on a provisional physico-chemical basis:

Specific:

(i) The attraction between protein products of chromomeres: long-range, unlimited. Zygotene and polytene.

(ii) The attraction between polymerized nucleic acid attachments of chromomeres; short-range, limited to pairs. Pachytene and perhaps polytene.

Non-specific:

(iii) The union of torsion which continues specific pairing along two threads when it ceases to be specific: the so-called non-homologous pairing. Pachytene especially in hybrids.

(iv) The fusion between the unpolymerized nucleic acid of all heterochromatin which has sometimes a false appearance of being specific at pachytene (or prophase of mitosis) because it also continues homologous pairing. It continues at metaphase as secondary and somatic pairing and acts at a distance.

On this basis pairing is an integration of protein and nucleic acid effects, free and fibrous proteins, dry and wet nucleic acids. On the other hand repulsion, at least of centromeres, seems to be a purely protein effect.

VII. CONCLUSION

These observations and speculations are, as I have said, merely first steps in rewriting cytology. But they show that in the chromosomes we have discovered how to make chemical processes structurally visible. These processes cover the physiological activities of chromosomes consisting of genes of four different types determining heredity and development, modified by differentiation and disease. They also cover mechanical activities which fall under the four headings of reproduction and spiralization, breakage and reunion. They further cover the joint mechanical and physiological activities which are bound up with chromosome pairing and with the relations of meiosis and mitosis in both of which the mechanical attributes of the chromosomes appear to depend on the physiological attributes of their genes. And finally they reach out toward the relations of viruses, plasmagenes, microsomes and the determinants of cancer.

The first fruit of a chemical approach to the genes and the chromosomes is to raise far more questions than it answers. The differences of nucleic acid behaviour between centromeres, nucleolar organizers and heterochromatin we partly understand. The underlying differences of protein structure still escape us. But so far as it does answer questions the consequence of the chemical method is, paradoxically, to give the subject greater biological solidity, greater predictiveness. This is especially obvious in regard to the integrated effects of radiation on the cell and the comparative study of cancer and blood diseases. At last these effects are beginning to make sense as a whole. And of course the solidity is not merely biological. We are now witnessing, after the slow fermentation of fifty years, a concentration of technical power aimed at the essential determinants of heredity, development and disease. This concentration is made possible by the common function of nucleic acid as the molecular midwife of all reproductive particles. Indeed it is the nucleic acids which, in spite of their chemical obscurity, are giving to biology a unity which has so far been lacking, a chemical unity.

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EXPLANATION OF PLATES

PLATE 1

Figs. 1, 2. Cold-starved metaphase chromosomes. Feulgen stain. Fig. 1. Pollen grain of triploid *Fritillaria pudica* with 18 chromosomes nearly all having multiple proximal segments of heterochromatin. Fig. 2. Root-tip of *Paris polyphylla* with 10 major chromosomes and one fragment, all except the fragment having single distal segments of heterochromatin. × 2000.

PLATE 2

Figs. 3–8. Nuclei at stages leading up to leptotene in different species of *Fritillaria*. Feulgen stain for desoxyribose nucleic acid. Figs. 3, 4. *F. lanceolata* (diploid) with central body of heterochromatin. Figs. 5–8. Species with no heterochromatin. Fig. 5. *F. sieheana*. Figs. 6–8. *F. obliqua*. Fig. 5. Pollen mother cell; the rest, embryo-sac mother cells. Figs. 3 and 5 show the earliest stage of nucleic acid concentration; figs. 4 and 6 show the partial dissipation of the dumps and the first signs of the charged chromosome threads; figs. 7 and 8, early and full leptotene with the gradual appearance of chromomeres and in fig. 8, of pairing. In all except figs. 5 and 7, the translucent refractive nucleolus can be seen from its effect on the focus of other structures. × 1000.

PLATE 3

Figs. 9, 10. Zygotene-pachytene in pollen mother cells of triploid (9) and diploid (10) *F. lanceolata*. Feulgen stain. In the triploid pairing is slower and never complete. In the diploid pairing is still incomplete at 12 o'clock. In the triploid the bodies of heterochromatin and the nucleoli are smaller and more numerous owing to lack of fusion. × 2000.

All preparations and photographs by Mr L. F. La Cour.

THE EXPERIMENTAL MODIFICATION OF NUCLEIC ACID SYSTEMS IN THE CELL

By P. C. KOLLER
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I. INTRODUCTION

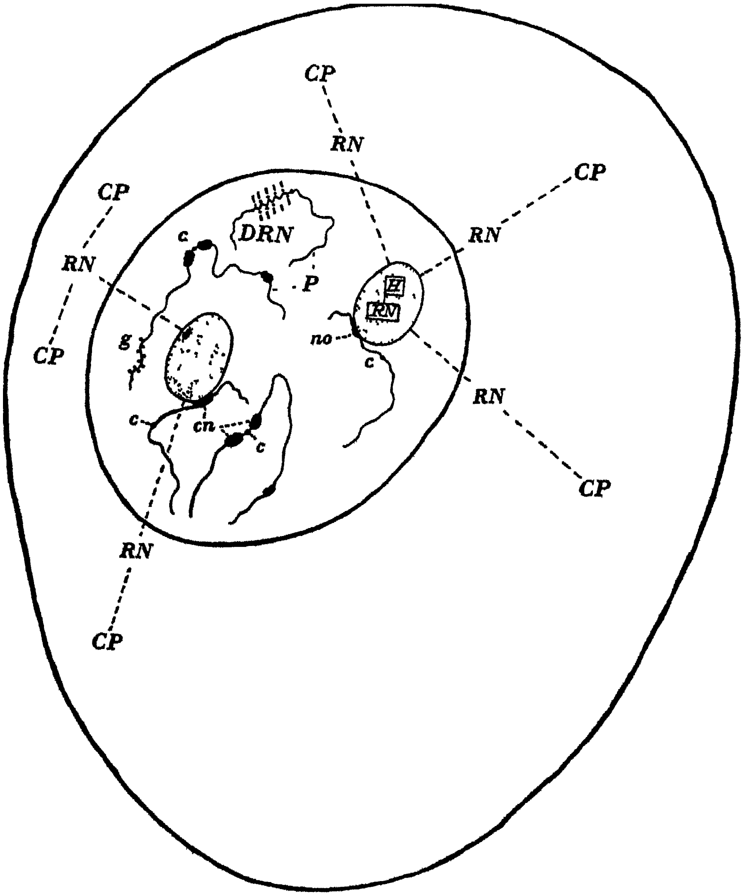
The concept of the organization of chromosomes in nuclear cytology is based on the work and interpretation of Caspersson (1936, 1941), Mazia & Jaeger (1939), Brachet (1940, 1944), Darlington (1942), and Claude & Potter (1943). These investigators, though differing in their experimental designs, came to the same conclusion, that the chromosomes during the mitotic cycle are composed of protein and nucleic acid of the desoxyribose type. The purpose of the present paper is, first to describe what I have seen in normal and abnormal cells under the effect of irradiation, chemical agencies and altered environmental conditions, and secondly, to interpret the changes induced in chromosome structure and in cell development in relation to protein synthesis and nucleic acid metabolism. Such an examination should help us in clarifying the chemical composition of the chromosomes and in understanding their role in the development, growth and differentiation of the cell.

II. CHROMOCENTRES AND NUCLEOLUS

During the cycle of mitosis, desoxyribose nucleic acid is attached at specific loci to the polypeptide chain which represents the permanent chromosome fibre. Maximum attachment or charge corresponds with the maximum spiralization of the chromosomes at metaphase of mitosis. The detachment of nucleic acid charge begins at late anaphase and proceeds through telophase. Its loss leads to simultaneous despiralization of the chromosome thread. The rate of nucleic acid detachment very often differs along the chromosome fibre. It is retained by specific segments of particular chromosomes which form the chromocentres of the resting nucleus. These centres give Feulgen-positive reaction (Text-fig. 1).

The chromosome regions which have a different cycle in respect of the nucleic acid charge from that of other regions are called heterochromatic to distinguish them from the others which are called euchromatic. The behaviour and reactivity of heterochromatin to specific factors differs from those of euchromatin and indicates differences in their structure. Caspersson (1941) assumes that heterochromatin produces simple protein, rich in hexone bases, and is built up of identical or similar elements. Darlington (1942) and Pontecorvo (1944) came to the same conclusion on purely cytological and genetical evidence.

It has been found that an intensive hyperfunction of the heterochromatin is characteristic of the cancer cells, and responsible for stimulating protein synthesis and nucleic acid metabolism (Caspersson & Santesson, 1942). Cytological analysis of tumour cells shows that all chromosome abnormalities and the increased rate of division itself can well be explained by assuming a quantitative change in nucleic acid synthesis (Koller, 1943*a*). I shall discuss in the following section the cytological mechanism which underlies the altered metabolic activity of cancer cells.



Text-fig. 1. Diagram showing the nuclear mechanism in relation to the protein forming system of the cell. *H*, histone; *RN*, ribose nucleic acid; *DRN*, desoxyribose nucleic acid; *CP*, cytoplasmic protein; *P*, polypeptide chain representing the uncoiled chromosome fibre; *g*, genes; *c*, centromere; *cn*, chromocentres; *no*, nucleolar organizer.

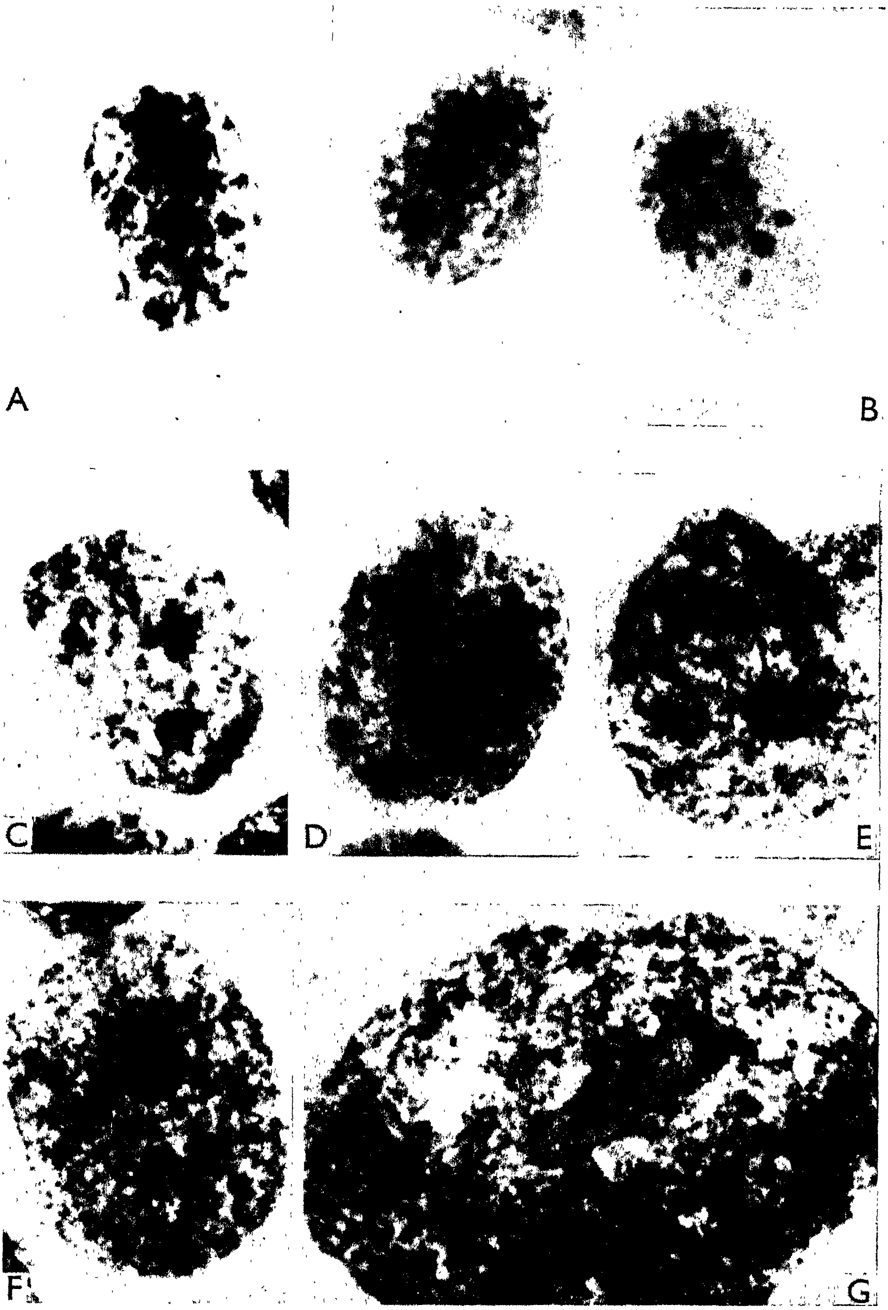
The maximum number of the chromocentres is found to be 36 in the resting nucleus of tumour cells in man (Pl. 1 A). They differ in size, indicating probably the difference in the length of the various heterochromatic regions. Occasionally two or more chromocentres may fuse together and form a larger aggregate. These centres are most numerous at the end of telophase, their number decreasing during resting stage. The release of the desoxyribose nucleic acid charge is slower and the process is much more gradual than it is in the euchromatic regions. It seems that the chromocentres act as regulators in the protein synthesis of the cell. The delayed detachment of nucleic acid must be responsible also for the reduced breakability of the heterochromatin, which was observed in root-tip cells of *Trillium* after irradiation with X-rays (Darlington & La Cour, 1945). When the nucleic acid charge of heterochromatin and euchromatin is the same as during resting stage, as it is in the sperm of *Drosophila*, both have the same degree of breakability (Kaufmann, 1939).

I have found that when two chromocentres are left in the resting nuclei of tumour cells, one or two nucleoli begin to develop from these centres (Pl. 1 D, E), hence they are considered as 'nucleolar organizers'. The nucleolus stains with methyl green-pyronin, which suggests the presence of ribose nucleic acid. This finding is similar to that which was reported previously by Caspersson (1941). During the very early stage of prophase when the chromosomes become distinct threads, the nucleolus can be seen lying amongst the chromosomes. It disappears in mid-prophase, well before the dissolution of the nuclear membrane. I found that the life of the nucleolus is very short in those particular tumour cells which are frequently undergoing division. When, however, chromosome synthesis is delayed or suppressed, the size of the nucleolus rapidly increases (Pl. 1 F, G). The cytoplasm of these cells is basophilic, as it stains with methyl green-pyronin. These cells are considered to be cells undergoing differentiation. The two cell types, one which is active and divides frequently, and the other which has a long resting stage or in which differentiation is completed, are linked together by many intergrades. They naturally make the identification of the two types often very difficult. Caspersson & Santesson (1942) were able to distinguish them in epithelial tumours by ultra-violet absorption spectroscopy (Text-fig. 2).

III. IONIZING RADIATION AND NUCLEIC ACID SYNTHESIS

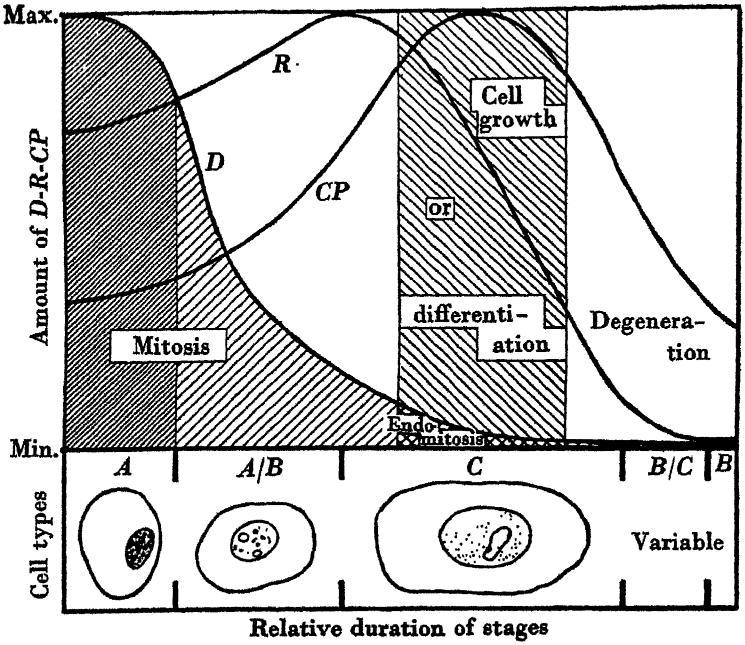
When tumour cells are irradiated with 100–300 r., it was found that 12–24 hr. after exposure the number of cells in mitosis is diminished. Even in those tumour regions which are healthy and richly supplied with food

PLATE 1



For explanation see p. 289

through a well-developed vascular system, the number of dividing cells show a decrease after radiation. I found that cytologically the cells are unaltered except that in the resting nucleus a nucleolus develops precociously (Pl. 1 B), and the cytoplasm of these cells stains with methyl green-pyronin. While the identification of the small nucleolus is very difficult because of the presence of numerous chromocentres in the nucleus in most of the cells, their basophilic cytoplasm can always be demonstrated.



Text-fig. 2. Diagram illustrating the cytological characteristics and the metabolic activity of the various cell types in tumour tissue. *A*, active, frequently dividing cell in resting stage; *B*, differentiating; *C*, degenerating cell types; *D*, desoxyribose nucleic acid; *R*, ribose nucleic acid; *CP*, cytoplasmic protein. The amount of desoxyribose nucleic acid determines the rate of mitosis.

The precociously formed nucleolus, the basophily of the cytoplasm in the potentially dividing cell type, and the suppression of mitosis suggest that 12–24 hr. after irradiation, with a dose of 100–300 r., the nucleic acid metabolism in these cells must have been altered. Mitchell (1942) suggested that irradiation inhibits the normal synthesis of thymonucleic acid by stopping the reduction of ribose nucleic acid, which would naturally account for the suppression of mitosis. The high concentration of ribose nucleic acid in the cytoplasm and particularly the precociously formed nucleolus which are observed a few hours after irradiation, indicate that irradiation is not only blocking the conversion of ribose nucleic acid but at the same time it increases the rate of nucleic acid production by means of stimulating the nucleolar organizing centres.

Ionizing radiation, besides acting directly upon the nucleus and cytoplasm, also acts indirectly on the nucleus through the cytoplasm or vice versa. This is the reason why the direct and indirect effects of the irradiation are not always distinguishable, especially when the analysis of these effects is carried out on cells or tissues which were exposed to a heavy dose of radiation. The two distinct processes: (*a*) the increased rate of production of ribose nucleic acid, and (*b*) suppression of mitosis, though often simultaneous, can, however, be separated when small doses (25–50 r.) are employed, or when the radiation is given at a dosage rate well below 1 r./min.

When pollen grains in *Tradescantia* were irradiated at 0·1 r./min. for 33 hr. 20 min. with γ-rays, the average number of dividing grains was about 37%, while in untreated anthers in the same developmental stage the percentage was much lower (20–25%). I have observed also that in the treated samples the rate of division is independent of the developmental stage of the anthers. By using a dose, not higher than 50 r., Darlington & La Cour (1945) found a slight stimulation in division frequency, which lasts 4–6 hr. after irradiation. A similar observation was made by Poulsen (1945) when round-cell sarcoma of mice was irradiated at low dosage rate (1 r./min.).

There is further evidence which suggests that the amount of nucleic acid is increased after irradiation. Darlington & La Cour (1945) found that the heterochromatin in root-tip cells of *Trillium* at low temperature is undercharged with nucleic acid. It seems that the amount of nucleic acid produced in the cell under this condition is not sufficient to satisfy the demand of all the chromosomes, and the heterochromatic segments are the first to suffer on account of the reduced supply. However, when such cells are irradiated, the nucleic acid starvation is wiped out. The only conclusion which can be drawn is that irradiation has increased the nucleic acid supply which can now satisfy the demands of all the chromosomes.

Amongst pollen grains which were kept for the greater part of their developmental cycle under uninterrupted radiation at low dosage rate, some were found with a much higher number of chromosome breaks than was expected (14 breaks against 1·27). Another abnormality observed in pollen grains irradiated at low dosage rate is the presence of several very small or minute fragments in some cells (Pl. 3A). These fragments are scattered in the cytoplasm or grouped close together in the equatorial plate, and stain like chromosome acentrics. These unexpected expressions of chromosome reactivity to radiation strongly suggest that by employing ionizing radiation at very low dosage rates, we may have succeeded in separating different responses in the various basic functions of nucleus, chromosomes and cytoplasm.

IV. IRRADIATION AND THE INTERACTION OF NUCLEUS AND CYTOPLASM

It was observed by several investigators (Marquardt, 1938; Koller, 1943*b*; Darlington & La Cour, 1945) that chromosomes in cells of divers organisms 2–6 hr. after irradiation clump and stick together at metaphase, and form bridges at anaphase and telophase. In *Tradescantia* pollen grains, stickiness is first seen 2 hr. after irradiation and it disappears 12 hr. after treatment with 360 r. at 20° C. Because sticky and non-sticky chromosomes are present together in irradiated cells, the stickiness cannot be due to change induced in the cytoplasm only. Darlington (1942) suggested that an excess amount of non-polymerized nucleic acid may be deposited on the chromosomes as a result of radiation. More investigations are needed, however, to determine the exact physical, physiological and biochemical basis of this specific and very universal primary reaction of chromosomes in irradiated cells. The data may help us to understand the basis of the inconsistency in the relative frequencies of X-rays, γ-rays, and α-rays in causing inhibition of mitosis, which is at present unexplained.

Table 1. *The time of appearance, greatest manifestation, and disappearance of primary radiation effects*

Radiation effects	Time of appearance in hr.		Time of greatest manifestation		Time of dis-appearance	
	360 r.	90 r.	360 r.	90 r.	360 r.	90 r.
Lack of orientation of chromosomes	1	3	3	3	12	9
Prolonged congression of chromosomes	2	3	4–5	3	48	9
Over-spiralization	3	7–8	5	—	12	12
Under-spiralization	5	5	—	—	7	—
Despiralization of the telomere region	3	—	—	—	7	—
Despiralization of the centromere region	3	—	—	—	—	—
Delayed division of centromeres	3	5	5	—	7	—
Stickiness of telomeres	2	7	7	7	48	—
Stickiness of chromosomes and chromatids	3	7	5	7	12	—
Altered orientation of spindle	7	—	—	—	24	—
Incomplete spindle	5	7	5	—	24	12
Abnormal vacuolation	12	—	—	—	24	—

The time of appearance, duration and degree of manifestation of the various primary effects in *Tradescantia* pollen grains following irradiation with 360 and 90 r. is given in Table 1. Some of the abnormalities which are very often observed together in the same cells are no doubt combinations of two or more different processes. Because most of these effects are related to chromosome behaviour, we are justified in assuming that the specific processes which underlie chromosome synthesis and organization are affected by the radiation. It is not improbable, however, that the primary effects of irradiation shown by the chromosomes may be only the end-result of a chain of reactions which have originated in the cytoplasm. The question is whether ionizing radiation with doses of between 200 and 500 r. can induce enzyme inhibition or alteration in the cytoplasm, or not.

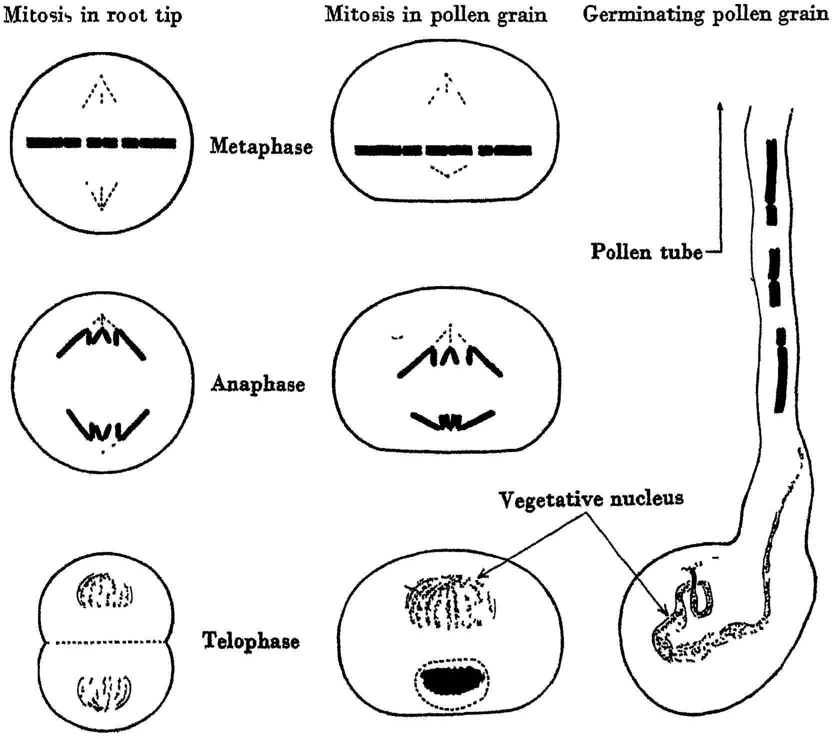
The experimental evidence at our disposal suggests that the enzymes of the cytoplasm are well protected from the effect of radiation (Dale, 1942). Recently further impressive evidence was provided by Whiting (1946). She exposed unfertilized eggs of *Habrobracon* to 42,000 r., as a result of which the chromosome mechanism of the female pronucleus is broken up completely and ceases to function. When, however, an untreated sperm nucleus is introduced into the heavily irradiated egg cytoplasm, cleavage division and segmentation followed normally. No radiation effects were detected on the untreated chromosomes of the male pronucleus in spite of the very heavily irradiated cytoplasm into which they were introduced at fertilization. The conclusion can therefore be drawn that the metabolic or enzyme activity of the cytoplasm is not impaired by the excessive dose of 42,000 r.

On the other hand, when the nucleus is damaged by irradiation and not replaced by a normal nucleus, cytoplasmic activity can be disturbed to such a degree that mitosis is greatly delayed and the cell may die without going through mitosis. I observed a few pollen grains of *Tradescantia* in which mitosis was delayed 7 days after irradiation with 360 r. The cells have shown precocious separation of sister chromatids at metaphase and a very high number of minute fragments or acentrics grouped together at one place, previously occupied by the resting nucleus. The long delay in mitosis is no doubt due to the great number of induced structural changes in the chromosomes, on account of which the nuclear control of cell metabolism has broken down. Though the interaction of the nucleus and cytoplasm is mutual, a great amount of the genetical and cytological evidence at our disposal clearly indicates that the nucleus is the primary focus of control in cell metabolism as it is manifested in development and differentiation (Darlington, 1944).

V. NUCLEIC ACID DISTRIBUTION AND DIFFERENTIATION

In dividing cells the equatorial plate lies between the poles. Its distance is the same from the two poles. If the metaphase chromosome is long, the proximal or centric region has a longer distance to cover from the equatorial place than the distal or telomeric region. In spite of this inequality in distances covered by different parts of the same chromosome, the rate of detachment of the nucleic acid charge and the despiralization of the chromosome thread during telophase is uniform all along the chromosome length, except in the heterochromatic regions.

The distance between the equatorial plate and the two poles, however, differs in pollen grains (Text-fig. 3). The chromosomes which will form the germinative nucleus travel less than half the distance which their sisters cover. Moreover, they do not show any decrease in the nucleic acid charge during telophase; on the contrary, their condensation increases and the length is shortened. This behaviour indicates that instead of the usual process of despiralization they undergo further contraction, and at the end of mitosis the chromosomes form the small germinative nucleus, which stains with Feulgen's basic fuchsin. Suita (1937) was the first to notice that while the sperm nucleus is very rich in nucleic acid, in the vegetative nucleus there is 'a remarkable decrease of thymonucleic acid'. During mitosis not only the hereditary material (e.g. genes) is distributed equally



Text-fig. 3. Diagram illustrating the distribution of nucleic acid by the chromosome mechanism. It is unequal in the daughter cells of pollen-grain division, because they differ in size. During pollen germination the vegetative nucleus is in a fluid state, the chromosomes within this nucleus lose their individuality.

between the daughter cells, but at the same time the mechanism of division guarantees the equal distribution of the chromosome nucleic acid too, which will be used in the two daughter cells during the rapid growth of the cytoplasm. In pollen grains the size of the daughter cells is unequal, and because one set of chromosomes retain their charge, only half of the amount of nucleic acid is available to satisfy the increased demand of the larger cell. The difference of reactivity to Feulgen's basic fuchsin of the germinative and vegetative nucleus indicates that in the latter desoxyribose nucleic acid is much more dispersed than in the former. The chromosomes of the vegetative nucleus do not organize themselves into independent entities again after the pollen-grain division has been completed, which suggests that they must have undergone a profound change in their structure.

The nucleus, however, in spite of this internal change still functions as a regulator of pollen germination. Its control can be eliminated only if the grains are irradiated before mitosis. Fragmentation of chromosomes of the future vegetative nucleus leads to the formation of micro- or super-numerary nuclei, and may also disturb the axis of division itself. In such pollen grains differentiation between vegetative and germinative nucleus is suppressed, and they fail also at germination (Koller, 1943 *b*). When, however, irradiation follows mitosis, germination is not prevented. The frequency of germinating grains is the same as in the untreated sample even in instances when a very high dose (10,000–30,000 r.) is used (Poddubnaja, 1936). The chromosomes of the pollen-tube division show primary as well as secondary changes (stickiness and breaks) which indicate that irradiation is effective on the germinative nucleus, while it is ineffective on the vegetative nucleus. The complete freedom of the latter from radiation-induced damage must be attributed to internal changes which have taken place in its chromosomes. It is a very tempting hypothesis to assume that on account of the complete detachment of the desoxyribose nucleic acid, the polypeptide chromosome fibre has broken down into its component units. This hypothesis is supported by the facts that the chromosomes never appear again, and that at the time of germination, or in the growing pollen tube, the vegetative nucleus is in a 'fluid state' without organized structure (Pl. 3 D). Its movement and shape are obviously determined by the strong cytoplasmic movement alone.

VI. THE NUCLEIC ACID SUPPLY WITHIN AND BETWEEN CELLS

Binucleate and multinucleate cells are frequent in various tissues of animals and plants. Mitosis as well as meiosis of the separate nuclei within the same cell was found to be synchronous, i.e. it takes place simultaneously. It is *to face p.* 275

PLATE 2

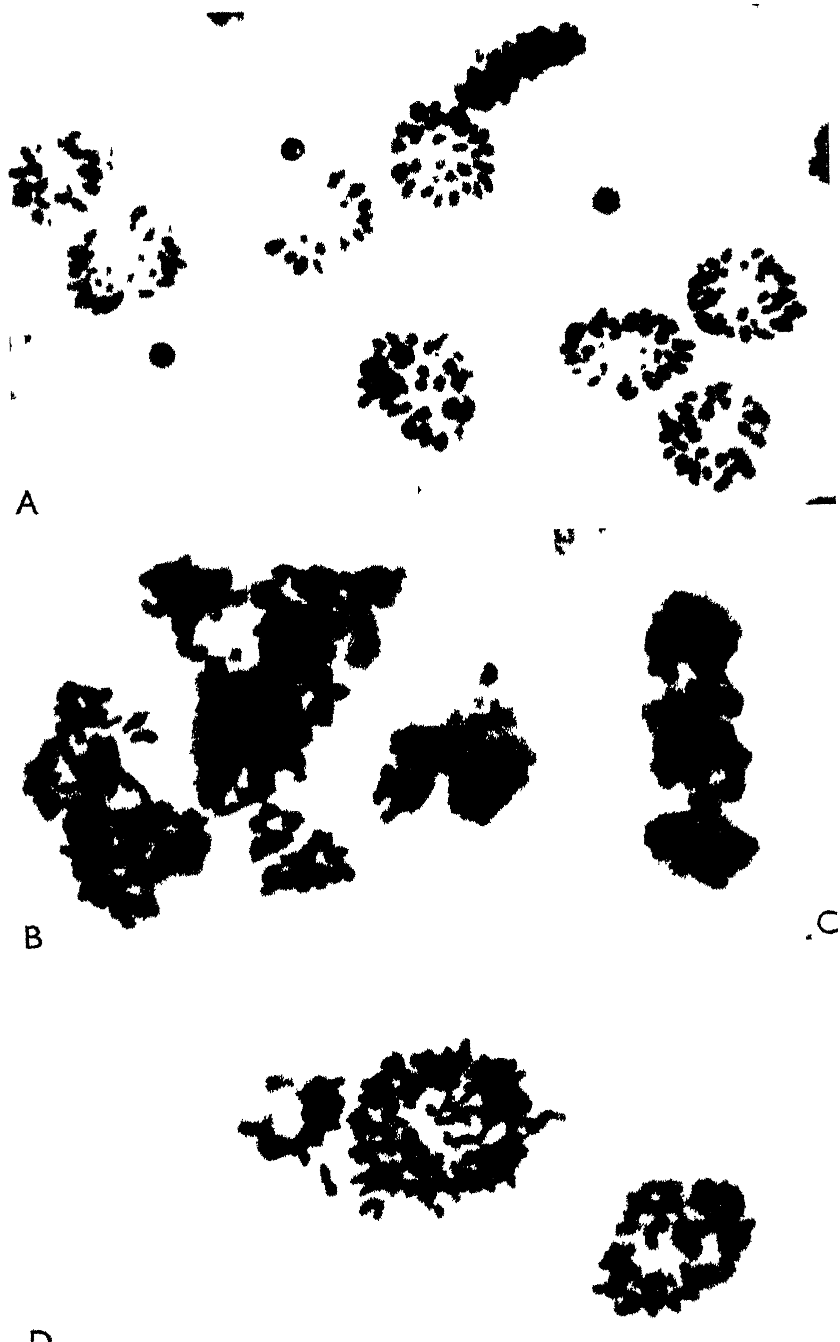
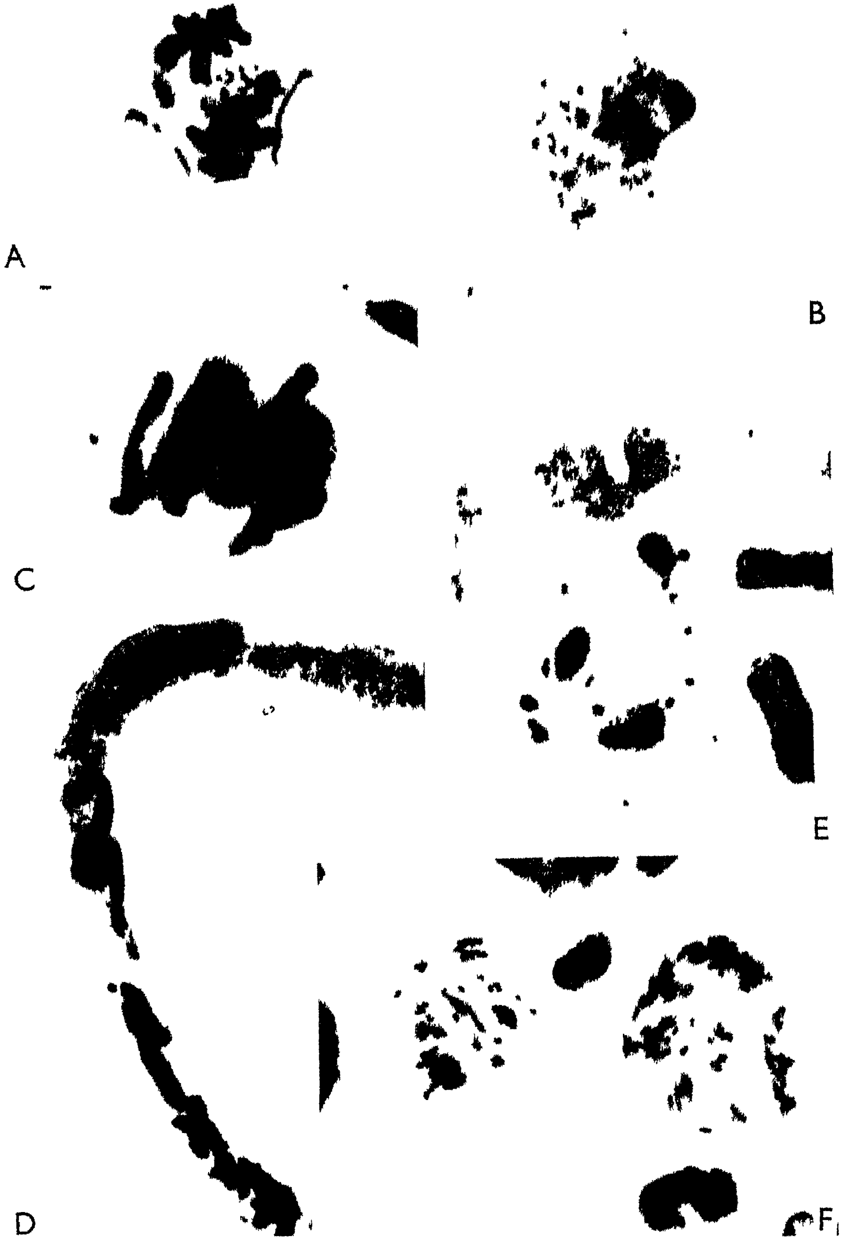


PLATE 3



For explanation see p. 289

the rule in multinucleate tumour cells (Pl. 2B, C), in megakaryocytes of human bone-marrow, in cells of growing root tips, in spermatocytes of insects and in mammals, etc. The simultaneous division of several independent nuclei can be understood only if we assume that the substance necessary for chromosome synthesis, or at least its precursors, are distributed throughout the cell. The centres of synthesis may be localized, but the even distribution of their product is guaranteed by a stable equilibrium between the demands of the individual nuclei.

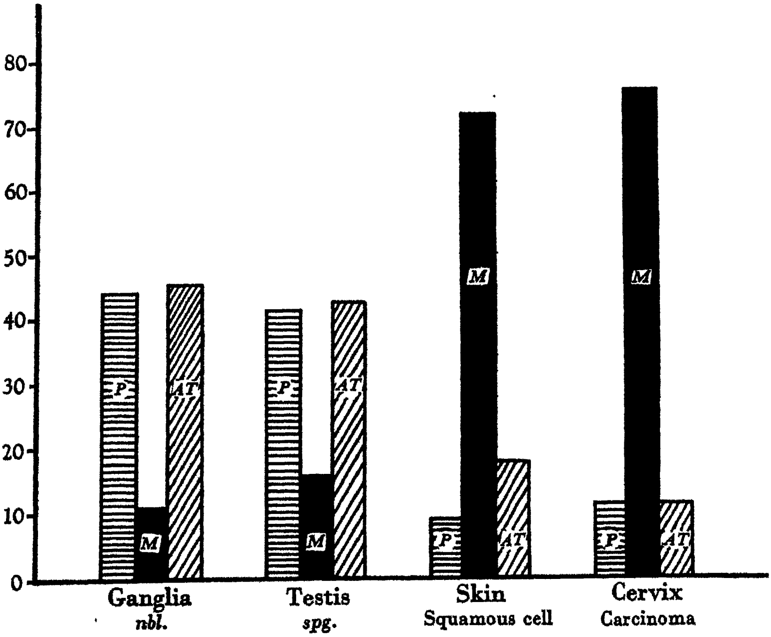
The cytological literature abounds in instances of synchronization of adjacent cells on a large scale, in diverse organisms and tissues such as the testes of insects, the anthers of orchids, the pre-spermatogonical cells of mammals, etc. (Pl. 2A), which shows that the precursors of chromosome synthesis which are produced in a cell can also be transferred to neighbouring cells. The diffusion of specific substances can be easily understood in cells of animal tissues, because there is no proper cell wall. We have, however, definite evidence that in cells of plant tissues, in spite of the well-developed cell wall, communication and mutual exchange of substances occur. Barber (1941) found that in *Uvularia* deficient pollen grains survive when they remain in contact with a normal grain. Due to this co-operation or transfusion of specific substances, the activity and development of the cells are synchronized, and they even exhibit the same abnormality in the formation of the spindle. Sax (1942) found two hypoploid pollen grains in *Tradescantia*, each with three chromosomes in mitosis. The grains were in close contact and he attributed the division of the two cells to the diffusion of cellular substances necessary for division. In cancer tissue I have often encountered cells with abnormally low chromosome numbers (12–32 instead of 48) in mitosis in company with other dividing cells (Pl. 2D).

VII. THE RATE OF NUCLEIC ACID SYNTHESIS AND MITOSIS

The deposition and polymerization of the nucleic acid on specific loci of the chromosome fibre takes place during prophase of mitosis. In some cells this process is long, taking perhaps several hours, while in others it is much shorter, taking a few minutes only. The duration of prophase indicates the rate of polymerization and attachment of desoxyribose nucleic acid, or perhaps the rate of conversion of ribose nucleic acid. It also indicates indirectly the amount of nucleic acid supply, which is available for chromosome synthesis. Thus a relatively short prophase suggests a rapid chromosome synthesis, which presupposes an abundant supply or a high rate of production of nucleic acids within the cell. Brachet (1940) and Painter

(1940) observed that the synthesis of cleavage chromosomes is very rapid in the segmenting egg of *Triton*, and found that the cytoplasm of the same egg is rich in ribose nucleic acid. The two phenomena are causally related. It seems that the amount of available nucleic acid is one of the chief factors which determines the rate of division.

The relative frequencies of mitotic stages may be taken as a measure of their relative duration. In normal tissues, it is the rule that the number of cells in metaphase is less than that of cells in prophase and post-metaphase.



Text-fig. 4. Diagram illustrating the percentage of mitotic stages in ganglia of *Cortophaga*, in testis and in tumours of man. *P*, prophase; *M*, metaphase; *AT*, ana-telophase; *nbl*, neuroblasts; *spg*, spermatogonial cells.

This was verified by observations on living cells. Wright (1925) found that in tissue cultures of fibroblasts, mitosis is completed in 31 min. 20 sec.: prophase took 14 min. 20 sec., metaphase 5 min. and post-metaphase 12 min. Barber (1939) reported that in *Tradescantia* staminal hairs, while prophase took 3 hr. 30 min.–4 hr. 30 min., metaphase took only 15–25 min.

I compared the frequency of the various mitotic stages in the ganglial cells of *Cortophaga* (Carlson, 1941) in the spermatogonial and tumour cells of man, and found that the proportion of the stages differs in normal and cancer tissues (Text-fig. 4). In the latter the relative number of cells in prophase is much less than that in the former, and we infer, therefore, that chromosome synthesis is extremely rapid in cancer cells. This fact, together with the frequent stickiness and clumping of chromosomes during metaphase, strongly suggests the view arrived at by Caspersson & Santesson (1942), that there is an excess and rapid nucleic acid production. I found also that occasionally the cytoplasm in dividing tumour cells stains with methyl green-pyronin. It is not improbable that a high concentration of nucleic acid in dividing cells is the cause of the delayed or completely suppressed spindle, a phenomenon which is very common in tumour cells.

VIII. EXTRA NUCLEAR SUPPLY OF NUCLEIC ACID AND RATE OF DEVELOPMENT

The cells in various tissues (e.g. root tip, anthers, testis, tumours, etc.) show different rates of development which are under external and internal control. The all-important cytological consequences of divergent or convergent development in the quantitative analysis of radiation effects were described by Darlington & La Cour (1945). They have shown that the mitotic cycle of pollen grains is accelerated as the number of dividing grains increases. Such an alteration in the rate of development was even more clearly seen in experiments when grains were irradiated at very low dosage rates (0·25–0·1 r./min.). In order to explain such an effect it is assumed that some specific substance must have been released and transmitted from cells which entered mitosis to cells not yet in division, which hastens their development. The developmental differences of cells can be levelled by intercellular co-operation. It is at its highest when behaviour and mitotic activity of cells are synchronized.

Basophily in the cytoplasm of pollen grains was found to appear first three days after division, at the time when the sperm nucleus is undergoing differentiation. This period also coincides with the dissolution of the tapetal cells of the anther. The sudden appearance of ribose nucleic acid in great concentration is very likely connected with the dissolution of the tapetal cells. Painter (1943) has shown that in *Rhoeo*, the pollen grains are bathed in a fluid substance which is derived from the tapetal cells. A similar phenomenon takes place during the development of the egg of *Drosophila*. The contents of the giant nurse cells with larger nuclei are absorbed into the cytoplasm of the egg. This may also explain the rapid rebuilding of cleavage chromosomes in the segmenting egg. It seems that the synthesis is in the nature of reassembling already existing materials, e.g. nucleotides, rather than actual synthesis of the building blocks from relatively simple substances. I observed that disintegration of chromosomes in dividing tumour cells with abnormally low chromosome number was associated with polymitosis of the adjacent daughter cell (Pl. 4A). The accelerated and repeated division led to an excessively increased rate of development throughout the tumour.

It may also be pertinent to the present discussion to point out that in animal tissues, the high number of cells in arrested metaphase after treatment with colchicine can be attributed to a similar process. The suppression of the anaphase spindle solely in those cells which would have entered division as a normal course cannot account for the high frequency of cells in metaphase. It seems that nucleic acid synthesis proceeds unhindered in the cells in which the division is arrested. The excess thus produced, on the one hand, is deposited on the chromosomes causing clumping and over-condensation from which the cell fails to recover; on the other hand, it is transmitted to adjacent cells. The cells which have gained nucleic acid, enter mitosis, but will be arrested at metaphase, as was the cell which acted as donor.

There is indirect evidence suggesting that nucleic acid supply may be influenced externally and also that cells might derive requirements from their surrounding medium. It was reported by La Cour (1944) that small or minute buds are extruded from the heterochromatic centres of the elongated and segmented nucleus in polymorphs and neutrophils of mice. A similar phenomenon was observed in man (Discombe, 1946). The concentration of nucleic acids in the cytoplasm, which is a characteristic property of these cells, may well be maintained by this mechanism. The significance of this process is shown by the fact that I found a great increase in the number of nuclear buds in mice which were kept 35 days on vitamin B-deficient diet. In spite of the increase in the number of nuclear buds the cytoplasm lost its basophilic property.

IX. HETEROCHROMATIN AND NUCLEIC ACID SUPPLY

The heterochromatic segments remain undercharged with desoxyribose nucleic acid, and underspiralized at metaphase of mitosis, when root tips of *Trillium* are exposed at 0° C. for a varying length of time (Darlington & La Cour, 1940). In chromosomes of animals, beside the cold treatment (Barber & Callan, 1943), food starvation has been found to reveal such segments (Wickbom, 1945). I observed, furthermore, that in the golden hamster not only nutrition (affected by diet and disease), but age and the season can also influence the amount of nucleic acid charge on the heterochromatic segment of the sex bivalent during meiosis (Pl. 3 C) (Koller, 1946).

It can be postulated that the rate at which nucleic acid metabolism is affected will be different for different tissues. In root tips of *Trillium*, after only 30 min. exposure at 0° C., nucleic acid starvation is said to be shown within the cell (Wilson & Boothroyd, 1941). In the liver cells of rats (Kosterlitz, 1944) one day on protein-free diet leads to 15 % reduction in the amount of nucleic acid and protein. This reduction was shown to be due to a fall in the amount of ribose nucleic acid in the cytoplasm (Davidson & Waymouth, 1944). The first effect of reduced diet on the heterochromatin in the spermatocytes of the hamsters was observed after 17 days.

The data obtained in various experiments indicate that when the amount of nucleic acid is diminished, a competition comes into operation for the available supply. Various levels can be distinguished in this competition: it may be (*a*) intrachromosomal, when the euchromatin competes with the heterochromatin of the same chromosome; (*b*) interchromosomal, when complete chromosome or chromosomes compete with each other, one being composed of heterochromatin (*Y* of *Drosophila*, the genetically inert *B*-chromosome of maize, supernumerary chromosomes in insects, etc.); (*c*) intercellular, when two or more adjacent cells compete, which is the case in the seminiferous tubuli of the hamster, and in tumours (Pl. 4B); and (*d*) intertissue, when cells of one tissue compete and have a greater demand for nucleic acid supply than cells of other tissues.

By analysing chromosome behaviour in the hamster under a greatly affected nucleic acid supply, it became apparent that chromosome pairing, crossing-over and post-pachytene association are determined by the amount of available nucleic acid. I found that extreme nucleic acid starvation is associated with the failure of pairing of the sex chromosomes and occasionally that of the autosomes. Because post-pachytene pairing of chromosomes depends on chiasma formation, which in turn depends on chromosome reduplication, it must be assumed that gene and chromosome reduplication is delayed, and crossing-over is prevented when the concentration of nucleic acid falls below the normal threshold. If the nucleic acid supply is not restored to the required level, the development of spermatocytes beyond pachytene stage may be permanently arrested, as was found to be the case.

The heterochromatin, on account of its specific structure and reactivity, behaves as an indicator of the metabolic conditions which prevail within the cell. But heterochromatin also functions as a regulator, being responsible for maintaining equilibrium between demand and supply for nucleic acid. The balance can be upset not only by external, but also by internal factors, e.g. by the addition of an extra heterochromatic chromosome to the normal chromosome complex. Such an addition was found to have been accompanied by an increase in the ribose nucleic acid content in the cytoplasm (e.g. in the egg of *XXY Drosophila* (Caspersson & Schultz, 1939)), and led to supernumerary division in *Sorghum* (Darlington & Thomas, 1941).

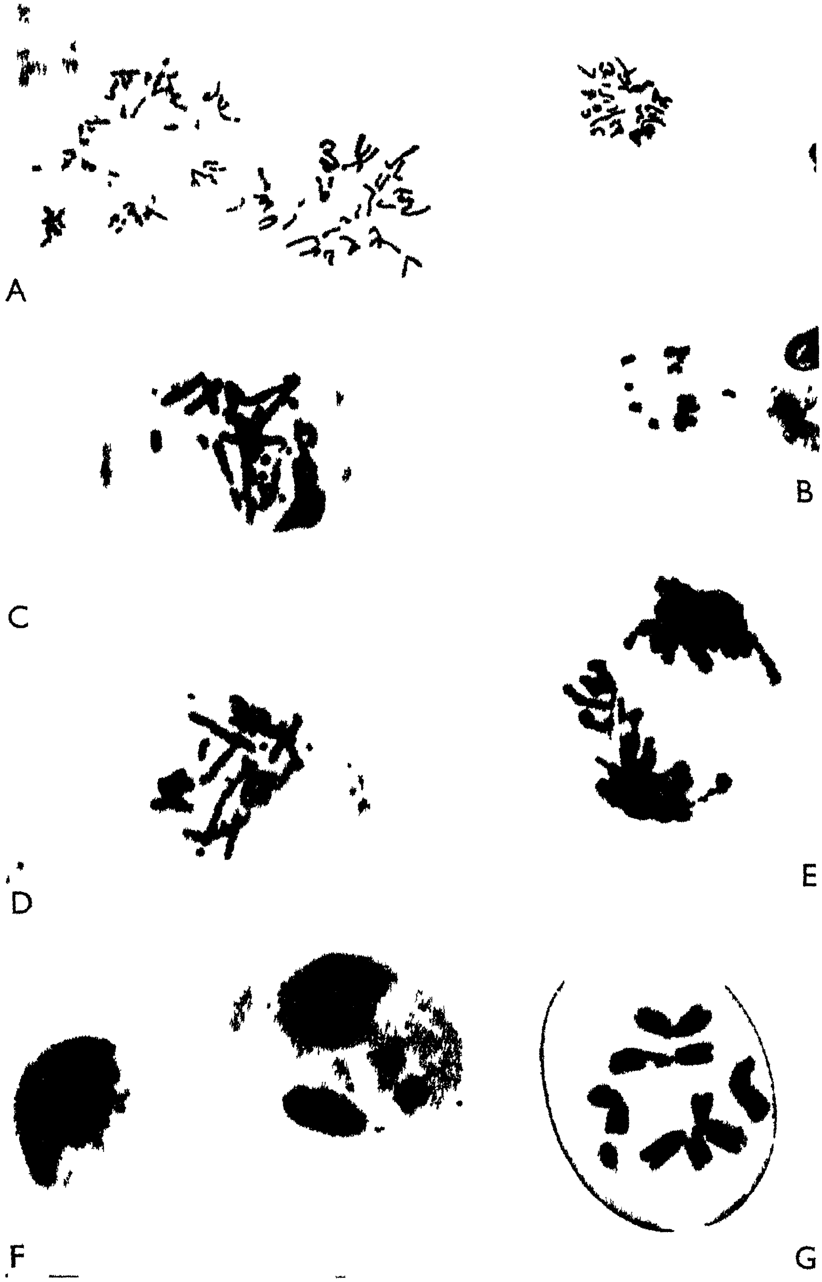
X. REACTIVITY OF CHROMOSOMES TO CHEMICALS

Most of the effects induced by chemical agencies are non-specific and are chiefly concerned with the inhibition or complete destruction of the cytoplasmic enzyme systems. The process usually is an irreversible one, resulting invariably in the death of the cell. The diverse changes which the chromosomes of the affected cells exhibit are brought about by very different processes, the complexity of which defied cytological analysis. In the following part I discuss the effects of a few chemicals only, which I found to affect chromosome structure and behaviour specifically.

An analysis of the effects has shown that the cytological consequences of chemical treatment are variable. They may be shown as (*a*) an increase in the basophily of the cytoplasm; (*b*) an increase in the size and number of nucleoli; and (*c*) alterations in the mitotic or meiotic chromosome behaviour and structure, such as spiralization, nucleic acid charge, breakage and reunion. These effects were observed in human tumours under treatment with stilboestrol, and in the Walker rat tumour under the influence of derivatives of 4-aminostilbene (Haddow, Harris & Kon, 1945). The effects induced by them in cells differ in detail. After treatment with stilboestrol the nuclear organizing centres were found to be under stimulation (Pl. 3 B). In some cells the nucleolus reached a very large size, and the cytoplasm became strongly basophilic. Fragmentation of chromosomes is very common. The fragments are often minute granules, and are scattered in the cytoplasm (Pl. 3 E). After treatment with 4-aminostilbene, the chromosomes break up and the spindle is either absent or abnormal (Pl. 3 F). Mitosis is incomplete and cells with nuclei of abnormal sizes and shapes are found. Because genetical evidence is lacking, the effects induced by stilboestrol and stilbene on the chromosomes may be interpreted as indirect effects which are brought about through disturbances in the cytoplasm.

The first chemical substance which could be assumed to affect chromosome structure directly was discovered by Auerbach (1943). She exposed adult male *Drosophila* to the vapour of $\beta\beta'$-dichlordiethylsulphide or mustard gas, and obtained in repeated experiments over 700 sex-linked lethals, several visible recessive and dominant gene mutations. Genetical tests carried out by her also indicated chromosome deficiencies, duplications, inversions and interchanges. In order to analyse the induced effects in the cell cytologically, I exposed anthers of *Tradescantia* to the vapour of concentrated, as well as diluted, mustard gas for 30 sec. to 7 min. (Koller, 1947). The first effect was observed 6 hr. after treatment, when the centromere of the chromosomes was affected and often accompanied by

PLATE 4



For explanation see p 280

partial uncoiling of the chromosomes themselves. The effect on the centromere is shown by misdivision which is particularly evident during the second meiotic division, 48 hr. after treatment (Pl. 4 E). The chromosomes show fragmentation to an extreme degree, the size of fragments varying very greatly (Pl. 4 C, F). Some of them are only minute granules, yet giving Feulgen-positive reaction. Regions can also be distinguished in the chromosomes which stain very slightly with Feulgen's basic fuchsin (Pl. 4 D). These particular segments, without desoxyribose nucleic acid charge, represent weak spots in the organization of the polypeptide chain, where the chromosomes usually break. Several treated pollen grains were encountered in the 48 hr. sample, in which the chromosomes form a deeply stained aggregate of varying sizes and shapes. By being clumped together into an amorphous mass, their individuality is completely obliterated (Pl. 4 F). The cell is unable to recover from such an over-condensation and consequently dies.

Very specific structural changes, including chromosome and chromatid (*B″* and *B′*) breaks, reunion of sister and non-sister chromatids, deficiencies, deletions and interchanges, were observed 2–5 days after treatment with mustard gas (Pl. 4 G). The initial changes in the chromosomes must have been induced within the resting nucleus. The end-results are similar to those produced by ionizing radiation. Though the actual breaks, produced by ionization in the peptide linkages of the chromosome fibre, may be due to chemical changes, yet it is difficult to visualize that the same reaction would be set in motion by a chemical substance. It seems rather more probable, and the cytological analysis also supports the view, that the latter inactivates specific loci by forming new combinations with the component units of the polypeptide chain, which make genes and chromosome regions unfit for reduplication or for polymerization of the desoxyribose nucleic acid.

The genetical and cytological analysis of gene and chromosomal changes induced by chemicals suggest a possible mechanism by which these processes might have originated in Nature. Their understanding is of paramount importance, because 'spontaneous' gene and chromosome mutation underlie variation and constitute the basis of evolution. We postulate that when a change occurs in external environment and before the organism has adapted itself to the change, the various and complex but interrelated chemical reactions within the cytoplasm of the cells are disturbed and altered. As a result of the faulty metabolism a new chemical substance is produced. This new substance may then be interpolated into specific reaction chains, e.g. nucleic acid synthesis, in which case one of the consequences might be expressed in genic or structural alteration of the chromosomes.

Abnormal development of cells with chromosome fragmentation could always be traced to deficient nutrition (La Cour, unpublished), or to sudden change in temperature (Darlington & Upcott, 1941). Deficient nutrition and change in temperature are obviously only the evocators of complex chain reactions within the cell, which reactions remain indeterminate. Chemical substances, on the other hand, do affect specific systems or components of a system; thus the end-effects may easily be predictable. Therefore, chemical compounds or their component units could be expected to be mutafacient evocators as effective as either temperature or ionizing radiation (Hadorn & Niggli, 1946).

The mechanism which is responsible for the effects due to chemical carcinogens can now also be visualized in its broader aspect. The carcinogens must act either directly or indirectly on those particular processes which underlie or regulate cell division and also chromosome organization. It is obvious that carcinogens must eventually affect the rate of mitosis, and it has recently been shown by Strong (1945) that they can also affect the chromosomes. He found that methylcholanthrene not only induced tumours, but morphological (e.g. coat colour) and physiological (e.g. growth rate) changes in mice, which had a genetical basis and have been transmitted by heredity. We may therefore infer that the change which becomes a permanent property of the cell is not merely a cytoplasmic change, but it involves, at the same time, the chromosomal or genic organization of the nucleus.

A new field has been opened up in experimental cytology by the use of chemicals. These substances which, when introduced into the cell, compete either for particular components of the various enzyme systems, or for specific nucleotides, are of special significance (Thomas & Drew, 1943; Gulland, Parsons & Barker, 1946). Our investigation, discussed above, can be considered a promising start in this direction. Research on similar lines will no doubt yield further information concerning the structure, organization and chemical composition of the chromosomes, and eventually may enable us to place gene mutation and cell proliferation under experimental control.

XI. CONCLUSION

It was shown that the various changes in chromosome structure and cell behaviour, which were observed under very different conditions, are all interrelated. The common denominator is the nucleoprotein nature of the chromosomes and the inference that they not only participate in nucleic acid synthesis but they also control it. Heterochromatin, on account of its specific structure and reactivity, behaves as an indicator of the metabolic conditions which prevail within the cell, because it seems to be responsible for maintaining the equilibrium between demand and supply for nucleic acid. Experimental evidence is presented which supports the view that chemical substances can be expected to be mutafacient agents as effective as either temperature or ionizing radiation.

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EXPLANATION OF PLATES

Plate 1

A. Nucleus of a resting tumour cell showing the numerous chromocentres which are Feulgen-positive in staining reaction. The resting stage is short and the cell undergoes mitosis frequently. (Feulgen's staining, × 2500.) B. Nucleus 12 hr. after irradiation with 300 r. showing six large chromocentres and the small precociously developed nucleolus. (Feulgen's staining, × 2500.) C. Nucleus with two chromocentres, which represent the nucleolar organizers. Cell with such nucleus will either enter mitosis or undergo differentiation. (Feulgen's staining, × 2500.) D, F. Nuclei with one nucleolus which develops from two nucleolar organizing centres. (Methyl green-pyronin staining, × 2500.) E. Nucleus showing two primary and two secondary nucleoli. The cell is undergoing differentiation. (Methyl green-pyronin staining, × 2500.) G. Nucleus of a fully differentiated cell. The enlarged nucleus suggests that the cell is polyploid. (Methyl green-pyronin staining, × 2500.)

Plate 2

A. Synchronous division of spermatogonial cells in the testis of the field-mouse (*Apodemus*). (Feulgen's staining, × 2200.) B. Synchronous division of 16 hypoploid nuclei in a giant cell from epithelioma. (Feulgen's staining, × 2200.) C. Polymitosis with synchronization in tumour cell from squamous carcinoma of the cervix. (Feulgen's staining, × 2200.) D. Synchronous division of three adjacent tumour cells; one is diploid, the others are polyploid and hypoploid. (Feulgen's staining, × 2300.)

Plate 3

A. Pollen grain of *Tradescantia* in anaphase 33 hr. 20 min. after continuous irradiation with γ-rays at 0·1 r./min. A large number of minute chromosome fragments are left on the equatorial plate. (Feulgen's staining, × 2000.) B. Nucleus of a resting cell from carcinoma of the breast showing the stimulation of the nucleolar organizing centres under treatment with stilboestrol. (Feulgen's staining, × 2200.) C. Sex bivalent of the golden hamster showing the under-spiralized heterochromatic segment. It is undercharged with desoxyribose nucleic acid. (Feulgen's staining, × 2200.) D. Pollen tube of *Tradescantia* showing the chromosomes of the germinative nucleus in pro-metaphase and the 'fluid' vegetative nucleus. (Gentian-violet staining, × 1800.) E. Fragmentation of chromosomes in tumour cell from carcinoma of breast under treatment with stilboestrol. (Lacmoid staining, × 1800.) F. Two cells from Walker carcinoma of the rat under treatment with 4-aminostilbene. The chromosomes are in a process of fragmentation, spindle is absent from the cells. (Feulgen's staining, × 1500.)

Plate 4

A. Chromosomes of two daughter tumour cells in metaphase. One set of chromosomes is undergoing fragmentation. (Feulgen's staining, × 1800.) B. Two dividing tumour cells showing competition for nucleic acid. The chromosomes of one cell are overspiralized and overcharged with nucleic acid as compared with the chromosomes of the other cell. (Feulgen's staining, × 1800.) C, D. Pollen grains of *Tradescantia* 48 hr. after treatment with mustard-gas vapour showing the fragmentation of chromosomes (C) and the detachment of the nucleic acid charge from sections of chromosomes (D). (Feulgen's staining, × 1500.) E. Misdivision of centromere 12 hr. after treatment with mustard-gas vapour.

(Pollen grain, Feulgen's staining, × 1500.) F. Two pollen grains 48 hr. after treatment with mustard-gas vapour. The division is completed in one grain, and the difference between the germinative and vegetative nuclei is very distinct. Two micronuclei and several nucleic acid granules are present in the pollen grain. The other cell shows an extreme degree of clumping of chromosomes. (Feulgen's staining, × 1500.) G. Pollen grain in metaphase 48 hr. after treatment with mustard gas. The structural changes induced by the treatment are chromosome and chromatid breaks and reunions and similar to those induced by ionizing radiation. (Feulgen's staining, × 1500.)

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